BREAST CANCER METASTATIC DORMANCY AND EMERGENCE, A ROLE FOR ADJUVANT STATIN THERAPY

by

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Breast cancer is responsible for the most new cancer cases and is the second highest cause of cancer related deaths among women. Localized breast cancer is effectively treated surgically. In contrast, metastatic cancers often remain undetected as dormant micrometastases for years to decades after primary surgery. Emergence of micrometastases to form clinically evident metastases complicates therapeutic intervention, making survival rates poor. The often long lag time between primary tumor diagnosis and emergence of metastatic disease motivates the development or repurposing of agents to act as safe, long term adjuvants to prevent disease progression.

The statin drugs have been FDA approved for the treatment of hypercholesterolemia for three decades. Despite their well-studied effects on lipid homeostasis, statins feature beneficial pleiotropic effects on other organ systems and pathologies. Several epidemiological studies have demonstrated statins do not affect cancer incidence but do reduce breast cancer mortality. Previous *in vitro* work has demonstrated growth-suppressive and apoptosis-inducing effects of statins in tumor cell lines. However, the mechanisms and markers that contribute to statin's suppressive effects on tumor cells remain unclear. We hypothesize that statins benefit cancer mortality by directly suppressing the outgrowth of dormant metastatic disease.

The data presented herein demonstrate that statins reduce tumor cell growth and survival, in part due to suppression of Ras and Akt signaling. Importantly, these effects are limited to the membrane-permeant lipophilic statins. In *ex vivo* and *in vivo* models of metastatic breast cancer,

statins reduce the proliferation at the site of metastasis but not the primary tumor. Tumor cells expressing membrane E-cadherin are relatively more resistant to statin therapy, which implies that the clinical cancer mortality benefit observed is due to decreased outgrowth of dormant epithelial micrometastases into clinically evident mesenchymal macrometastases. These data motivate the further study of statins as safe, long term adjuvants to prevent emergent breast cancer. Moreover, these data recommend a therapeutic switch to lipophilic statins in breast cancer patients taking statins for other diseases. That statins selectively suppress metastatic but not primary tumor growth is promising for their use as safe, long term adjuvants to reduce recurrence and mortality from metastatic breast cancer.

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PREFACE

ACKNOWLEDGEMENTS

This thesis is a compilation of my work as a graduate student in the department of Cellular and Molecular Pathology (CMP) at the University of Pittsburgh. Large portions of this thesis have been previously published in the following references[1–4], and will be restated prior to the sections to which this pertains. On personal and professional levels, graduate school is challenging in that it demands learning new skills and coping with the tumultuous ups and downs of a rigorous research training program. I would not be where I am today without the guidance and support of my mentors, classmates, friends, and family.

First, I would like to thank my thesis mentor, Dr. Alan Wells, for his persistent support and guidance over these past three years. I was fortunate to have been introduced to Alan by a mentor of mine from my undergraduate years at MIT, Dr. Linda Griffith (whom I am also lucky to have on my thesis committee). When I first met Alan, I felt an instant connection. He is one of the most knowledgeable people I have ever met and has the remarkable trait of always finding a way to smile and laugh. Even when discussing serious topics or failed experiments, Alan always would look rationally and optimistically towards the next step. The number of times he has taught me life lessons, not only those related to research, is astounding. I think his approach to mentorship is so successful because it focuses on the big picture rather than the minute details. Alan was never the mentor to constantly look over my shoulder and make sure I did the experiments needed. Our weekly meetings most often discussed the larger picture of the research projects underway rather than forming a checklist of experiments to perform. As an organized person who feels more comfortable with accomplishing lists of tasks rather than making them, this was difficult for me at

first. But overtime, I felt myself becoming more independent in the lab. Instead of feeling the need to constantly ask advice from Alan or other graduate students and post docs in his lab, I felt confident in my ability to design goals and experiments to answer the research project's objectives. At the same time, whenever I did struggle with details of a particular experiment, Alan was always there to help. The balance Alan achieved between providing support and allowing independence allowed me to blossom as a graduate student.

The primary goal I had for my PhD was to develop myself as a researcher and build skills that I would need to succeed in research. Working under the tutelage of Alan allowed me to develop all of those skills. While still a second year medical student, after completing a ten week lab rotation, Alan motivated me to write an F30 grant, which was eventually funded my first year of graduate school. Alan has taught me how to design, write, and submit manuscripts, which will be invaluable in my future career. The research projects I undertook with Alan allowed me to learn a wide variety of laboratory techniques, from basic cell culture, to microfluidic *ex vivo* systems, to working with mice. I feel lucky to have gained experience in so many aspects of research during my PhD years. Also, I am incredibly thankful for Alan allowing me to spend one day per week doing clinical activities over the entirety of my PhD. As an MD himself, I feel he understood and demonstrated how one can be a successful physician scientist. More than anything, I am lucky to have been guided by such a considerate mentor. Alan does not demand, he does not order. He inspires.

The second group of people I would like to thank are my wonderful dissertation committee members: Drs. Donna Beer Stolz, Zoltán N. Oltvai, Partha Roy, Kari Nejak-Bowen, and Linda Griffith. Through our biannual committee meetings, I was able to present my work and receive valuable feedback with progressing my project, both big picture and experimental help. A large

portion of my thesis uses different imaging techniques to collect and analyze data, and Donna's help in that area has been invaluable. I have been grateful for Zoltán getting me involved with this thesis project initially during my first lab rotation with Alan. Moreover, Zoltán's persistence to involve me with similar, including a more computationally-aimed project, has widened my breadth of knowledge. Having never worked with mice before, Kari's comments regarding experimental design and blood collection have been extremely helpful. Partha has advised me on experiments to do, both during committee meetings and weekly data club that is shared between his lab and Alan's. Finally, I am eternally grateful to Linda for having been a mentor of mine since my undergraduate years at MIT, introducing me to Alan in the first place, and continuing to bring experience and enthusiasm to the table. You all have been such meaningful mentors and I look forward to staying in touch over the years to come.

Third, I would like to thank all of the people whom have helped me with experiments and learning techniques over my several years in graduate school. Drs. Sarah Wheeler and Amanda Clark took me under their wing early on, and taught me a plethora of techniques, from basic cell culture to advanced liver tissue culture in a microphysiological system. Drs. Bo Ma and Hanshuang Shao have been extremely helpful in teaching me transfection and western blotting, among other skills. Diana Whaley has been invaluable with her experience and assistance with mouse experiments and ordering reagents. I would also like to thank Tony Green at the Shadyside Histology Core for processing my mouse tissue and all of the people at the Center for Biological Imaging (CBI) at the University of Pittsburgh for their help, in particular Morgan and Callan. Finally, I would like to thank my CMP program director Wendy Mars and administrator Amanda Bytzura, and MSTP director Dr. Richard Steinman and administrator Phuong Pham for their ongoing support.

Fourth, I would like to thank my clinical mentors who continue to inspire me. Dr. Wallis Marsh first introduced me to hepatobiliary surgery and continues to be a supportive mentor. Dr. Kurt Weiss taught me a lot about orthopedic oncology, which combines my interests in orthopedics and cancer, and has been a pleasure to work with and learn from. Dr. Weiss continues to be an inspiration to me as a funded surgeon scientist. Finally, I would like to thank Dr. Gary Gruen for his mentorship over the past four years. Gary has been more than just a mentor to me, he has been a friend. He has helped me develop my future career plans and has introduced me to clinical research, which I see myself pursuing as a surgeon scientist. He has been a big advocate for my future and has invested a lot of his time into teaching me valuable lessons, for which I am eternally thankful.

Finally, I would like to thank all of my friends and family for their never ending support and encouragement. Whenever I was having a bad day in lab, Ahmad, Drew, and Kyle would always help cheer me up. I'm thankful for conversations with my MSTP classmates, especially Aaron, Mike, Heather, and Colleen, which always reminded me that I had friends whom could empathize with my experiences. I'm incredibly thankful for everyone in the Pitt Med School PalPITTations, for always bringing such light and cheer to our weekly Tuesday night rehearsals, and truly feel I have made friends for life in that group. I would like to thank my friend Edgar, who has been a best friend of mine for the past 7 years starting at MIT, for the times we would hang out and talk about research, life, and other topics. You always managed to joke about things and brighten my spirits, even when I wasn't feeling the best. I would also like to thank Carly, who has been so loving and supportive this past year. Our copious outings downtown, to restaurants, to concerts, to food- or drink-based events, festivals, and the trips we have taken have been amazing experiences that reminded me that life exists outside of lab. Whenever I have struggled, you have

always been so supportive and found a way to make me smile. Finally, I would like to thank my parents for their undying support. Mom, thank you for always taking my phone calls (even when you were teaching) and telling me that I could do it. Having completed a PhD yourself, you gave me such great advice to stick with it and that it would all work out. Whenever I wasn't getting great data, you were so supportive and reminded me it took you several years to get your first data. Dad, thank you for all of your advice and support during these tough years. You always said you believed in me and helped me believe in myself. Even though you are always busy, you would stay on the phone as long as I wanted to talk. I'm grateful to you both, Mom and Dad, for your eternal patience, advice, and support.

It is impossible to recognize everyone who has had a positive impact on my life and PhD training these past several years. I feel truly lucky to have such an amazing group of mentors, classmates, and loved ones, and look forward to our relationships growing in the years to come.

ABBREVIATIONS

5-FdUMP	5-fluorodeoxyuridine monophosphate
5-FU	5-fluorouracil
5-FUTP	5-fluorouracil triphosphate
A1AT	Alpha-1 antitrypsin
ADCC	Antibody-dependent cellular cytotoxicity
ADH	Atypical ductal hyperplasia
AFP	Alpha-feto protein
AI	Aromatase inhibitor
Akt	Protein kinase B
ALD	Alcoholic liver disease
ALDH	Aldehyde dehydrogenase
ALH	Atypical lobular hyperplasia
ALT	Alanine aminotransferase
AMPK	AMP-activated protein kinase
Ang-2	Angiopoiten-2
AP-1	Activator protein 1
ASCSM	All site cancer-specific mortality
ASCVD	Atherosclerotic cardiovascular disease
AST	Aspartate aminotransferase
AUC	Area under the curve
BAK	Bcl-2 homologous antagonist killer
BCSM	Breast cancer-specific mortality
BFS	Biochemical free survival
BMI	Body mass index
BRCA1	Breast cancer 1
BRCA2	Breast cancer 2
Cap	Capecitabine
CCLE	Cancer cell line encyclopedia
CDK	Cyclin dependent kinase
CETP	Cholesterol ester transfer protein
Cis	Cisplatin
CK	Creatine kinase
CKs	Cytokeratins
Cpa	Cyclophosphamide
Crb	Carboplatin
CTR1	Copper transporter 1
CWRT	Chest wall radiotherapy
DAMP	Damage-associated molecular pattern
DCIS	Ductal carcinoma in situ
dCTP	Deoxycytidine triphosphate
dFdU	Difluoro-deoxyuridine
DHEAS	Dehydroepiandosterone sulfate
DMSO	Dimethyl sulfoxide
Dnr	Daunorubicin

Dox	Doxorubicin
DPD	Dihydropyrimidine dehydrogenase
dTMP	Deoxythymidine monophosphate
Dtx	Docetaxel
Ecad	E-cadherin
E-cadherin	Epithelial cadherin
EdU	5-Ethynyl-2'-deoxyuridine
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
eNOS	Endothelial nitric oxide synthase
EPC	Endothelial progenitor cell
Epi	Epirubicin
ER	Estrogen receptor
ErbB	Epidermal growth factor receptor
ERE	Estrogen response element
ER-EIA	ER monoclonal enzyme immunoassay
Erk	Extracellular signal-related kinase
ESC	Embryonic stem cell
FAS	Fatty acid synthase
FBAL	α-fluoro-β-alanine
FPP	Farnesyl pyrophosphate
GEF	Guanine exchange factor
Gem	Gemcitabine
GGPP	Geranylgeranyl pyrophosphate
GGT	Gamma glutamyl transferase
GI	Gastrointestinal
Grb2	Growth factor receptor-bound protein 2
GSK3	Glycogen synthase kinase 3
Gys	Grays
H&E	Hematoxylin and eosin
HB-EGF	Heparin binding EGF
HCC	Hepatocellular carcinoma
HDL	High density lipoprotein
HELU	Hyperplastic enlarged lobular units
HER	Human epidermal growth factor receptor
HER2	Human epidermal growth factor receptor 2
HER3	Human epidermal growth factor receptor 3
HER4	Human epidermal growth factor receptor 4
HMG-CoA	β-Hydroxy β-methylglutaryl-CoA
HMGCR	HMG-CoA Reductase
HPF	High powered field
HSC	Hepatic stellate cell
HSP90	Heat shock protein 90
HUVEC	Human umbilical vein endothelial cell
IC_{50}	Half-maximal inhibitory concentration

Ida	Idarubicin
IDC	Invasive ductal carcinoma
IGF-1	Insulin growth factor 1
IGF1R	Insulin growth factor 1 receptor
IHC	Immunohistochemistry
IL	Interleukin
ILC	Invasive lobular carcinoma
IM	Intramuscular
IP	Intraperitoneal
iPSC	Induced pluripotent stem cell
IV	Intravenous
Jnk	c-Jun N-terminal kinase
KC	Kupffer cell
K_i	Mean inhibitory concentration
Ki-67	Marker of proliferation Ki-67
LAMPS	Liver acinus microphysiology system
LCIS	Lobular carcinoma in situ
LDL	Low density lipoprotein
LDL-C	LDL cholesterol
LDLR	LDL receptor
LECM	Liver extracellular matrix
LET	Linear energy transfer
LHRH	Luteinizing hormone releasing hormone
LPS	Lipopolysaccharide
LSEC	Liver sinusoidal endothelial cell
LVEF	Left ventricular ejection fraction
MAPK	Mitogen activated protein kinase
MDR1	Multi-drug resistance 1
Mek1/2	Mitogen activated protein kinase kinase
MErT	Mesenchymal to epithelial reverting transition
MFP	Mammary fat pad
MGM	Mixed graphical model
mir	microRNA
MMP-9	Matrix metalloproteinase-9
MPCC	2D micro-patterned co-culture
MPS	Microphysiological system
mTORC	Mamalian target of rapamycin complex
Nab-Ptx	Nanoparticle albumin bound paclitaxel
NCI-60	National cancer institute 60 cell lines
NER	Nucleotide excision repair
NF-κB	Nuclear factor kappa beta
NK	Natural killer cell
NPC	Non-parenchymal cell
NRG	Neuregulin
NSCLC	Non-small cell lung cancer
NTCP	Sodium-taurocholate co-transporting polypeptide

OATP	Organic anion-transporting polypeptide
P53	Tumor protein p53
PARP	Poly-ADP-ribose polymerase
PBS	Primary biliary cirrhosis
PCSK9	Proprotein convertase subtiliskin/kexin type 9
PCSM	Prostate cancer-specific mortality
PD-1	Programmed death-1
PDK1	Phosphoinositide-dependent kinase 1
PD-L1	Programmed death-ligand 1
PFS	Progression free survival
PHH	Primary human hepatocyte
PI3K	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIP_3	Phosphatidylinositol 3,4,5-triphosphate
PR	Progesterone receptor
PSA	Prostate-specific antigen
PSC	Primary sclerosing cholangitis
PTB	Phosphotyrosine-binding domain
PTEN	Phosphatase and tensin homolog
Ptx	Paclitaxel
Quad	Quadriceps
Rb	Retinoblastoma
RFB	Radial flow bioreactor
RFS	Recurrence free survival
ROCK	Rho associated protein kinase
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
S1P	Site-1 protease
S2P	Site-2 protease
Sb-Ptx	Solvent-based paclitaxel
SCAP	SREBP cleavage-activating protein
SCLC	Small cell lung cancer
SERD	Selective estrogen receptor degrader
SERM	Selective estrogen receptor modulator
SH2	Src homology 2
siRNA	Short interfering RNA
SLCO	Solute carrier organic anion
SMA	Smooth muscle actin
Sos-1	Son of sevenless homolog 1
Sp-1	Specificity protein 1
SRE	Sterol response element
SREBP-2	Sterol regulatory element-binding protein 2
STAT3	Signal transducer and activator of transcription 3
SVM	Support vector machine
TCGA	The cancer genome atlas
TDLU	Terminal ductal lobular unit

T-DM1	Trastuzumab emtansine
TGFα	Transforming growth factor alpha
TIL	Tumor infiltrating lymphocyte
CYP450	Cytochrome P450
TNF	Tumor necrosis factor
TNM	Tumor, Nodal, Metastasis
TopII	Topoisomerase II
tPA	Tissue plasminogen activator
T-ReCS	Tree-guided recursive cluster selection
TS	Thymidylate synthase
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
ULN	Upper limit of normal
UTP	Uracil triphosphate
WBRT	Whole breast radiotherapy
ZEB1	Zinc finger E-box binding homeobox 1

1.0 BREAST CANCER PATHOPHYSIOLOGY AND THERAPY

1.1 INTRODUCTION

Breast cancer is a significant cause of morbidity and mortality in the United States, with over 250,000 new diagnoses and 40,000 deaths per year from the disease. The work herein demonstrates the feasibility of repurposing members of the statin drug class to combat metastatic breast cancer recurrence, the primary cause of breast cancer mortality, using the liver as a model organ system for metastasis. The first three chapters of this dissertation provide background material to frame these investigations. First, important aspects of breast cancer, including clinical and molecular designations and therapy, are discussed. Second, a historical and mechanistic perspective on the statins, including their use in cancer, is presented. Finally, liver physiology and relevance as a model for metastatic disease are introduced.

1.2 CLINICAL EPIDEMIOLOGY

Breast cancer is the most prevalent cancer and the second highest cause of cancer-related mortality in women and will be responsible for an estimated 266,120 new cases and 40,920 deaths in 2018[5]. Importantly, only about 1% of new breast cancer cases and deaths occur in men, making this disease one almost entirely of the female gender[6]. In addition to these new cases, approximately 1

63,410 cases of breast carcinoma in situ will be diagnosed, some of which will progress to invasive disease[6]. One in eight women can be expected to be diagnosed with invasive breast cancer in her lifetime[6]. Breast cancer carries the highest financial cancer-associated healthcare burden with an estimated cost of $16.5 billion in 2010[7]. This cost is projected to increase to $20.5 billion by 2020, the highest percentage increase in cancer spending (32%) aside from prostate cancer (42%)[7].

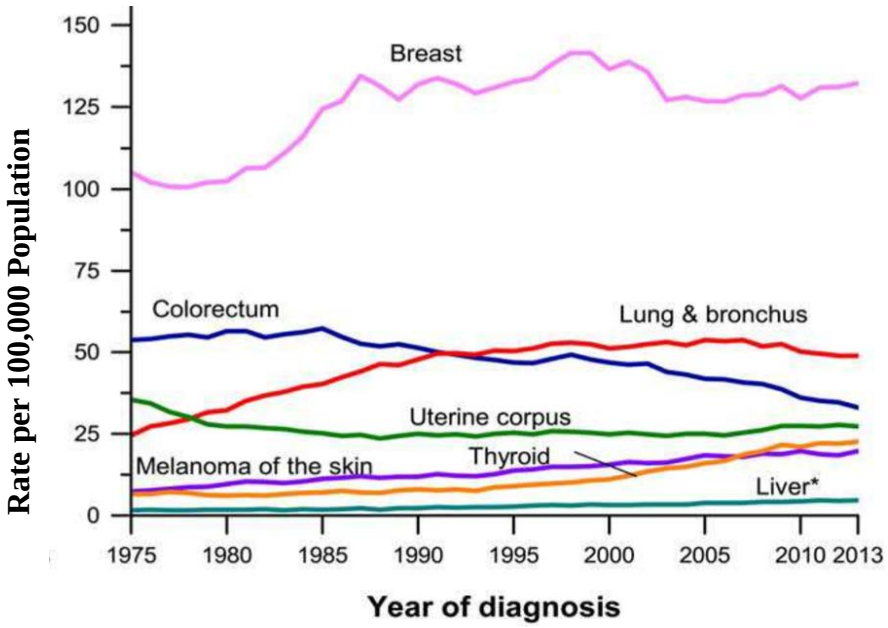


Figure 1. Female cancer incidence

Female cancer incidence between 1975 and 2013. The increase in incidence in breast cancer from 1980-1987 is due to increased mammography screening. Breast cancer remains the most commonly diagnosed non-skin cancer in women. Data are open access and modified from Cancer Statistics 2017[6].

The incidence of breast cancer has been steadily increasing by 0.4% per year in the general population. This rate of increase in breast cancer incidence has been stable except during the period of 1980-1987 (Figure 1), due in large part to the significant rise in screening mammography that

resulted from the 20-30% decrease in mortality due to screening mammograms observed in Swedish randomized trials in the 1970s and 1980s[8–10]. The rate of death from breast cancer has decreased by approximately 1.5% per year since 1989, with the mortality rate in 2014 being 21.2 per 100,000[6] (Figure 2).

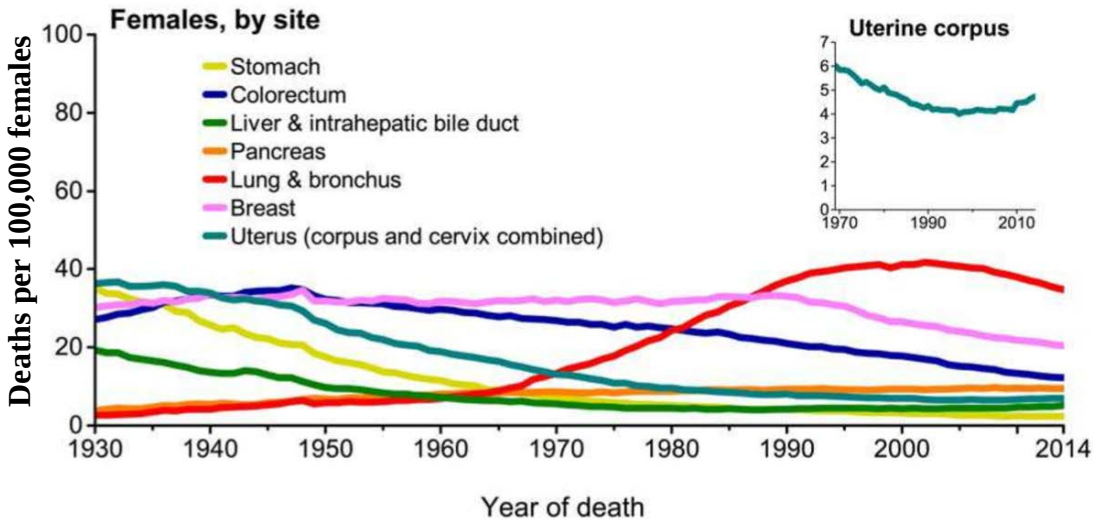


Figure 2. Female cancer death

Female cancer deaths between 1930 and 2014. The female specific death rates for breast, colorectal, and lung cancers have decreased by 1.5%, 1.3%, and 1.4% per year since their inflection point of descent, respectively. Data are open access and modified from Cancer Statistics 2017[6].

Only 6% of primary diagnoses involve metastatic (distant) disease (Figure 3), and therefore the decrease in breast cancer mortality must be primarily due to better treatment of localized and, especially, regional disease[6,11]. The five year survival rate for localized disease increased from 97% to 99% in two decades, while the corresponding survival rate for regional disease showed a larger increase from 76% to 85% (Figure 3). While survival rates for metastatic disease improved

from 20% to 26% over the same two decades (Figure 3), the continuing significantly lower survival rates demonstrate the need for therapeutic strategies that benefit patients with metastatic disease.

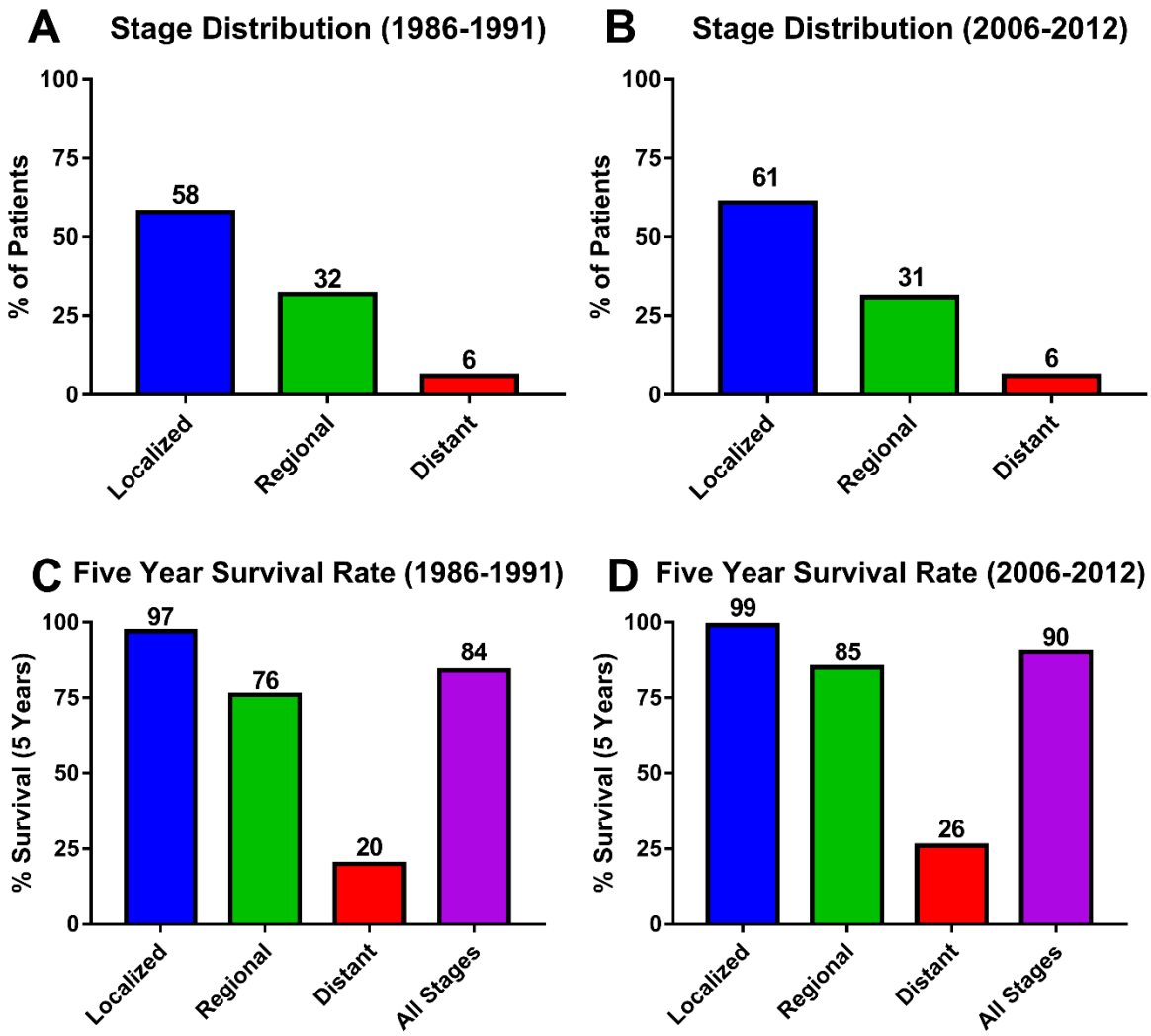


Figure 3. Breast cancer stage distribution at diagnosis and associated mortality rates

(A,B) Breast cancer stage at patient diagnosis and (C,D) five year survival rate for various stages of breast cancer from data published in (A,C) Cancer Statistics 1996 and (B,D) Cancer Statistics 2017. Figures were constructed using data provided in these references[6,11]

Many factors influence a woman's susceptibility to developing breast cancer. Women have a higher risk of developing breast cancer with increasing age, with an incidence plateau occurring in a woman's 7th decade[6,12]. Women whom have earlier onset of menarche and later onset of menopause are at a higher risk for breast cancer, which is likely due to higher cumulative estrogen exposure[12,13]. Indeed, other studies have linked postmenopausal obesity (increased estrone production from adipocytes) and long term estrogen replacement therapy to an increased breast cancer risk, primarily associated with hormone positive breast cancers[14,15]. Conversely, earlier age of first full term pregnancy is associated with a decreased risk of breast cancer, likely due to differentiation of mammary cells to a more quiescent phenotype following parturition[16,17]. While the use of oral contraceptives increases the risk of breast cancer, their use also decreases the proportion of tumors found upon diagnosis that have spread to sites outside the breast[18].

Several genetic factors influence the risk for developing breast cancer. The main breast cancer syndromes involve loss of a tumor suppressor gene. The most common genes involved in hereditary breast cancer are BRCA1 and BRCA2. These genes increase risk for breast cancer in an autosomal dominant fashion and account for roughly 5-15% of all breast cancer diagnoses, with a higher prevalence observed in women with asynchronous contralateral breast cancer[12,19,20]. The cumulative risk for developing contralateral breast cancer with a BRCA1 or BRCA2 mutation is almost 50% by 25 years after diagnosis of the first breast cancer[21]. Other common genetic diseases that dramatically increase the lifetime risk of breast cancer, in addition to other cancers, are Li-Fraumeni syndrome (p53 mutation) and Cowden syndrome (PTEN mutation), which will not be further discussed. For a more comprehensive discussion of genetic conditions and genes that predispose women to an increased risk of breast cancer, the reader is directed to other references[22–24].

In addition to estrogen exposure and genetic factors, lifestyle decisions can also impact breast cancer risk in women. As mentioned above, obesity increases risk for breast cancer both in disease incidence, tumor burden, and higher grade of tumors[25]. Higher estrogen levels have been found in women with higher BMI, suggesting estrogen plays a role in increasing breast cancer risk in obese women. Adipokines, such as leptin and adiponectin, have also been implicated in breast cancer development[26,27]. Alcohol consumption also increases breast cancer risk in women even at exposure as low as three drinks per week[28,29]. As with obesity, it has been proposed that alcohol consumption raises breast cancer risk by increasing endogenous estrogens secondary to decreasing liver catabolism of sex steroids[30]. Finally, low physical activity also increases the risk for breast cancer and is more significant for women with BMI less than twenty five[31,32]. Physical activity is thought to reduce breast cancer risk through decreasing body fat, insulin levels, adipokines, and as a result, hormone levels[32,33].

In conclusion, breast cancer is the most common and second most mortal cancer in women (after lung cancer). Several lifestyle and genetic factors can predispose women to the development of breast cancer, including BRCA1/2 mutations, obesity, estrogen exposure, alcohol use, and physical activity. While mortality from breast cancer has decreased by 38% over the past 25 years, the survival rate in distant breast cancer remains low. These epidemiological data motivate the development of therapeutic strategies that can extend the survival of patients with metastatic breast cancer.

1.3 DEVELOPMENT, ANATOMY, AND PHYSIOLOGY

The development, anatomy, and physiology of the breast are all important for the development of primary cancer and its eventual metastatic spread. The human female breast begins developing as early as 4 to 6 weeks of gestation and continues developing into adulthood[34,35]. Breast tissue consists of both epithelial and mesenchymal (stromal) elements, which are organized into a branching ductular system ending in the terminal ductal lobular unit (TDLU) (Figure 4). The branching of breast ductules is modest until their rapid expansion via side branching in women under the influence of sex hormones that accompanies thelarche[34–36]. The breast ductular structure further develops during and after pregnancy and is responsible for decreasing breast cancer risk in women after parturition.

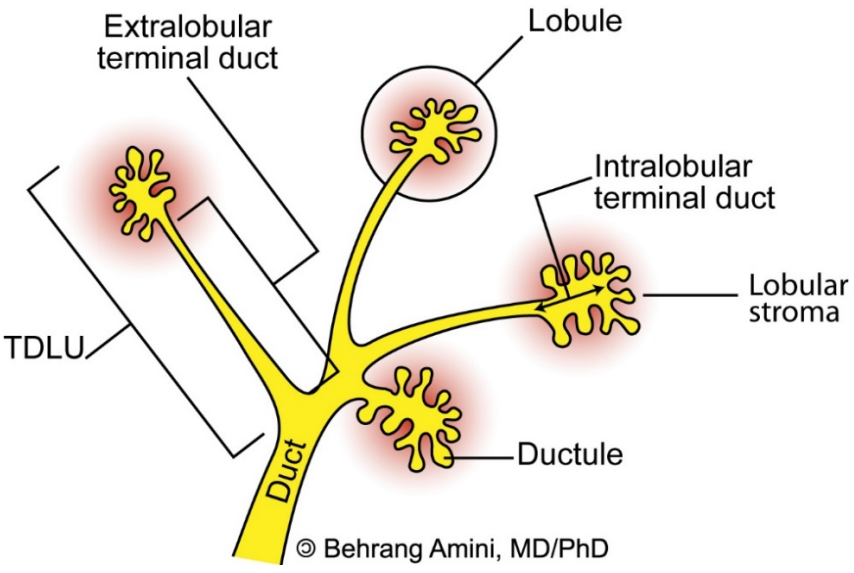


Figure 4. The terminal ductal lobular unit of the breast

The breast is organized into a branched network of ducts ending in a terminal ductal lobular unit (TDLU). This image is copyrighted by Dr. Amini as indicated, has not been changed, and its free use has been allowed by the terms of the Creative Commons Attribution-Noncommercial-Share Alike 3.0 license.

The female breast lies on the anterior thoracic wall, deep to the dermis and superficial to the pectoralis major fascia[37]. Approximately 10-15% of the breast is composed of epithelial tissue, whereas the remainder comprises stromal elements. The breast is anchored to the skin by the suspensory ligaments of Cooper, whose contraction (by malignant cancers or tissue fibrosis) can cause dimpling of the skin[37]. The blood supply entering the breast consists of branches of the internal mammary artery, axillary artery, and posterior intercostal arteries. The venous drainage follows the arteries to the axilla, with branches of the internal thoracic vein serving as the highest contributors to flow[37]. The lymphatic drainage to the breast is more than 75% to the axillary lymph nodes, with the medial aspect of the breast instead draining to the parasternal lymph nodes or deep cervical nodes[37]. Importantly, lymph nodes are both a common route for the metastatic spread of breast cancer and are one of the most significant prognostic marker for patient survival[37,38].

Normal breast physiology, especially in the context of puberty, pregnancy, lactation, and menopause is quite complex and will not be discussed in its entirety. To summarize, after pubertal breast development, a cyclical microscopic proliferation and regression of breast tissue occurs during the menstrual cycle. During pregnancy, the breast develops in preparation for lactation after parturition and is fully developed after only 16 weeks gestation[35]. This development consists of three main phases: lactogenesis (the start of milk secretion), lactation (sustained milk secretion), and involution (epithelial apoptosis and reversion to a pre-pregnant morphology)[35]. Importantly, the differentiation that accompanies lactation results in a decreased risk for pre-menopausal breast cancer[39,40]. For a comprehensive review of breast physiology, the reader is directed to more complete references[35,41].

1.4 DISEASE PROGRESSION

Breast cancer is a complex entity, with stages encompassing the range from the benign breast carcinoma in situ to highly invasive metastatic breast cancer. The loss of tumor suppressor genes and/or presence of oncogenes catalyzes the progression between phases. This process is commonly referred to as the metastatic cascade and involves the spread of aggressive tumor cells from the primary organ (breast in this case) to distant sites through the circulatory system. The rest of this section provides a brief review of the metastatic cascade and its implications for survival, as is relevant for the work contained herein.

The metastatic cascade begins with tumor growth at the primary tumor site and ends with metastatic growth at a distant site. The steps are illustrated in Figure 5 and summarized in Table 1 below. The metastatic cascade begins with the growth of tumor cells in the breast to form a breast carcinoma in situ that is initially constrained by the basolateral basement membrane of the TDLU[42]. Tumor progression through the basement membrane and into the surrounding environment allows for dissemination by two distinct modes. First, local invasion commences when a tumor cell achieves at least partial separation from the primary mass. The process continues when this cell reorganizes and migrates through surrounding tissue to arrive at an adjacent site as a cohesive expansion of the primary mass[43,44]. Second, tumor cells can disseminate to distant organs through complete separation from the primary tumor, invasion through the surrounding tissue, and intravasation into a conduit (lymphatic or vascular)[43]. Importantly, this stage often involves a transition from an epithelial phenotype, in which E-cadherin expression facilitates cohesion between neighboring cells, to a mesenchymal phenotype, in which E-cadherin loss facilitates cellular elongation and dissociation from neighboring cells[44,45]. This transition is called an epithelial to mesenchymal transition (EMT).

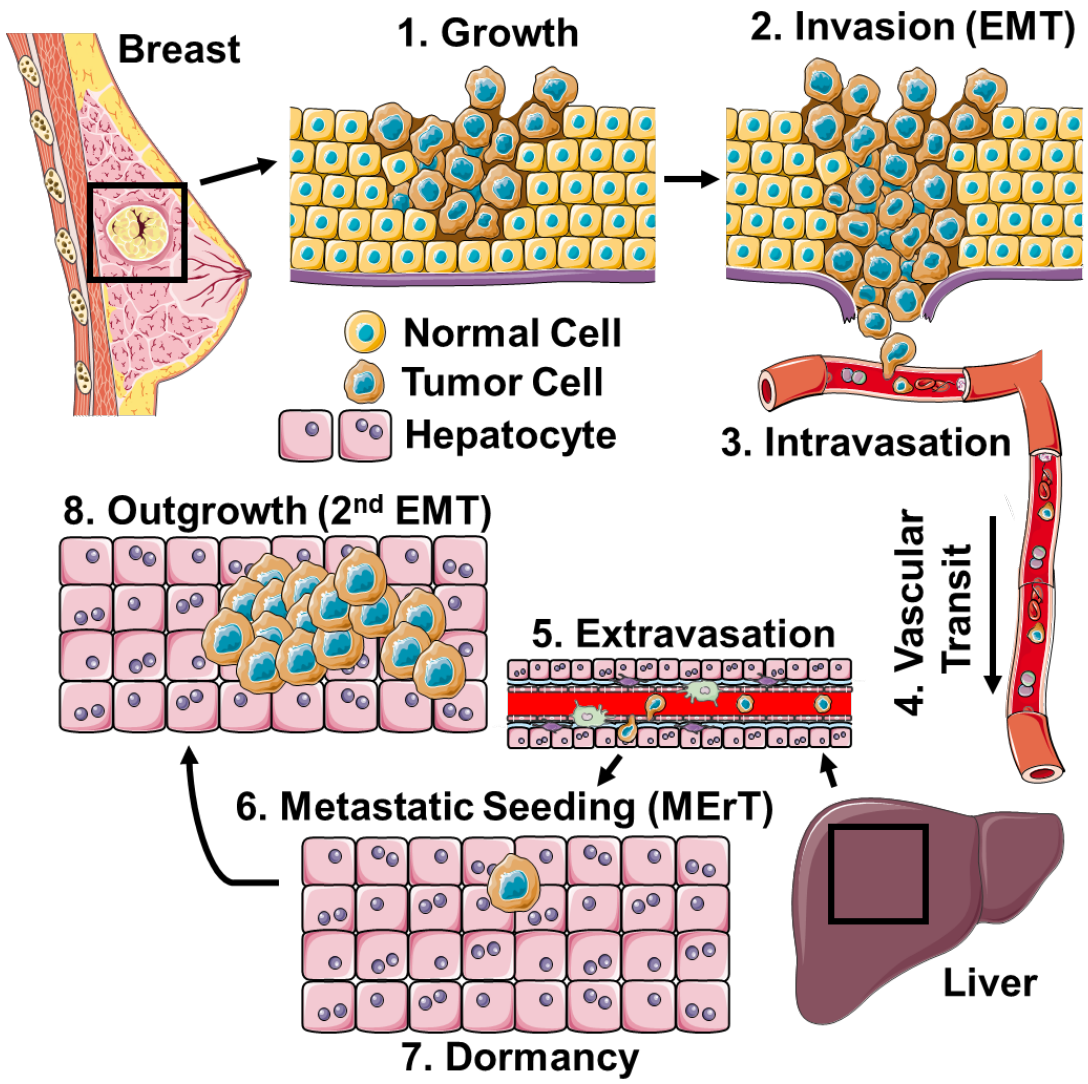


Figure 5. A simplified schematic of breast cancer metastasis to the liver

(1) Primary breast tumors grow and undergo (2) epithelial-to-mesenchymal transition (EMT), in which they lose E-cadherin and subsequently invade through the basement membrane. (3) Invading tumor cells intravasate into nearby vessels, (4) travel through the vasculature to the liver, and (5) extravasate into the surrounding parenchyma. (6) Tumor cells seed in the liver parenchyma as micrometastases by undergoing a mesenchymal-to-epithelial reverting transition (MErT) involving the re-expression of E-cadherin. (7) Seeded tumor cells next enter a period of dormancy. After years to decades, (8) dormant tumor cells undergo a secondary EMT and outgrow to form macrometastases. This diagram was created using images from Servier Medical Art[46].

Table 1. The stages of the metastatic cascade

Step	Description
Growth	Proliferation at the primary tumor site, confined by the basement membrane
Invasion	Extension through the basement membrane, often accompanied by EMT (loss of E-cadherin)
Intravasation	Invasion into a lymphatic or vascular conduit
Vascular Transit	Survival in the vasculature from shear stress, immune attack, and survival factor depletion
Extravasation	Recognition of endothelial cells at a distant site and invasion through the vascular basement membrane into the tissue parenchyma
Metastatic Seeding	Integration into the local tissue parenchyma, facilitated by MErT (re-expression of E-cadherin)
Dormancy	A period of quiescence lasting years to decades
Outgrowth	Microenvironmental signals stimulate a second EMT (loss of E-cadherin) and proliferation to form a clinically detectable metastasis

Tumor cells need to accomplish all steps in order to form an overt, clinically detectable metastasis.

EMT = epithelial to mesenchymal transition; MErT = mesenchymal to epithelial reverting transition[44,45,47,48].

Following cell intravasation into the vasculature, the tumor cells must survive transit to the distant organ site and recognize resident endothelial cells[44]. Of note, lymphatogenous metastases eventually drain into the vasculature through the left subclavian vein. This phase of the metastatic cascade is the most difficult for tumor cells due to the lack of supportive survival signals from the tumor microenvironment, immune surveillance, and presence of high shear stresses[44,48]. Upon recognition of endothelial cells at the distant site, tumor cells must extravasate and integrate into the surrounding parenchyma. This is facilitated by tumor cell plasticity, as breast carcinoma cells often re-express E-cadherin upon metastatic seeding, facilitating their integration into the surrounding parenchyma and providing survival signals[44,45,49]. This transition is called a mesenchymal to epithelial reverting transition (MErT). Tumor cells often remain in a quiescent state after metastatic seeding, termed “dormancy,” which can last years to decades after metastatic seeding[44,48]. The last stage of the metastatic cascade is outgrowth of tumor cells at the distant site,

likely due to stimulatory factors from the microenvironment[44,47,48]. This phase is accompanied by a second EMT, as E-cadherin loss is characteristic of large metastases[49].

Metastasis is the largest predictor for breast cancer morbidity and mortality, with only 10% of patients surviving ten years beyond discovery of their metastasis[50]. Moreover, metastases are often undetected at initial presentation, in particular when not involving the bone, as these often result in more presentable symptoms like as hypercalcemia or pathologic fractures (Table 2)[51]. Whereas rates of bone metastases in breast cancer patients are comparable between initial presentation and autopsy, rates of lung and liver metastases are 4 to 15 fold higher at autopsy (Table 2)[50], indicating that the issue is more of detection than presence, and that the bone, liver, and lung are most likely all involved as end sites for the metastatic cascade during initial dissemination.

Table 2. Prevalence of breast cancer metastases

Metastasis Site	At Presentation (%)	At Autopsy (%)
Bone	40-75	44-71
Lung	5-15	59-75
Pleura	5-15	23-51
Liver	3-10	56-65
Brain	<5	9-20

Prevalence of breast cancer metastases at initial presentation and upon autopsy. Data were taken from Jolly et al[50].

Table 3. Primary causes of death from metastatic breast cancer

Cause of Death	% Total	Commonly (% Subclass)	% Due to Mets
Pulmonary Insufficiency	26	Metastases (65%) Restrictive Disease (31%)	65
Infection	24	Pneumonia (58%)	N/A
Cardiac Failure	15	Heart Failure (29%) Pericardium (29%) MI (21%)	38
Hepatic Failure	14	Metastases (100%)	100
CNS failure	9	Meninges (40%) Brain (40%)	80
Hemorrhage	9	Organic (47%) DIC (20%) Local Tumor Invasion (13%)	13%

Primary causes of death from metastatic breast cancer as a clinicopathologic investigation. Data were taken from Hagemeister et al[52]. Mets = metastases, CNS = central nervous system, Organic = unrelated to disease or treatment, DIC = disseminated intravascular coagulation.

Patients with metastatic disease have poor survival. The metastases themselves are responsible for approximately half of all metastatic breast cancer deaths[52]. Other significant primary causes for death from metastatic breast cancer are infection, hemorrhage, and hepatic and central nervous system failure[52]. These data are summarized in Table 3. That liver failure results in 1 in 7 deaths from breast cancer is surprising, as the liver has a larger reserve capacity than most other organs. This highlights the involvement of the liver in breast cancer metastasis.

In summary, breast cancer progression to metastatic disease is accomplished through the metastatic cascade, which begins with primary tumor growth and ends with emergence of dormant metastases to form clinically evident metastases. Once a patient has metastatic disease, her prognosis is poor. The extent of metastatic disease is often underestimated until autopsy, in particular in the liver and lung. The most common causes of death from breast cancer are pulmonary insufficiency, infection, and failure of the heart, liver, and central nervous system.

Inhibition or delay of the steps of the metastatic process is desirable to reduce the mortality from breast cancer.

1.5 CLINICAL AND MOLECULAR PATHOLOGY

Breast cancer can be classified pathologically by three main systems: histological, clinical, and molecular. The histological classification of breast cancer refers to the morphology observed on classical hematoxylin and eosin stained slides of tumor samples. The clinical classification of breast cancer refers to a combination of tumor stage and grade. Finally, the molecular characterization of breast cancers refers to a combined assessment of histological appearance and expression of markers such as estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). The next several sections expand on each of these three classification systems in turn.

1.5.1 Histologic Classification of Breast Cancer

There are currently 17 discrete histological classifications for breast cancer[53]. The majority of cancers are classified as invasive ductal carcinoma (IDC), which accounts for approximately 75% of tumors[53,54]. Invasive lobular carcinoma (ILC) comprises 15% of all breast cancers, and the remaining 10% of tumors can be classified into multiple subtypes including apocrine carcinoma, adenoid cystic sarcoma, metaplastic carcinoma, micropapillary carcinoma, and tubular carcinoma[53–55]. The two most common types of breast cancer, IDC and ILC, which together account for 90% of all breast cancers, are discussed briefly below.

Invasive ductal carcinoma is the most common malignant neoplasm of the breast. First reported almost fifty years ago, the initial stage of ductal carcinoma of the breast is marked by regions of hyperplasia and carcinoma in situ developing from the terminal ductal lobular unit (TDLU)[56,57]. It is important to note that the word "stage" in this context refers to the histological organization of the tumor cells and not to the clinical stage, to be discussed below. This initial stage is followed by ductular unfolding, distension, and subsequent union of multiple smaller ductules to form larger oviform lesions[57]. The modern terminology for these various stages, in order, is: 1) hyperplastic enlarged lobular units (HELU), 2) atypical ductal hyperplasia (ADH), and 3) ductal carcinoma in situ (DCIS)[58]. All of these stages are classified as premalignant without any obligation for progression. However, once cancerous cells invade across the basement membrane into the surrounding tissue, the pathology is then classified as invasive ductal carcinoma[42]. Morphologically, ductal carcinoma cells resemble the ductal epithelium in that they are moderate to large in size, columnar in appearance and organization, and are frequently polarized[59].

Lobular carcinoma follows an identical progression scheme to that of ductal carcinoma with similar terminology. For example, atypical lobular hyperplasia (ALH) and lobular carcinoma in situ (LCIS) are equivalent stages of ADH and DCIS respectively. As lobular carcinomas emerge from the lobular cells in the TDLU, they appear morphologically similar to their physiological counterpart. Namely, lobular carcinoma cells are relatively smaller in size than ductal carcinoma cells, are non-polarized, and are cuboidal in appearance and organization[59]. Due to the lack of cohesion between cells, lobular carcinoma cells typically invade the stroma in a 'single-file' manner[60]. This lack of cohesion is most commonly due to lack of E-cadherin expression, which is absent in 90% of invasive lobular carcinomas (ILC), but present in almost 100% of IDC[61,62]. The

incidence of ILC has increased more rapidly than that of IDC, which is concerning, as the former is more difficult to detect by both mammography and physical exam[63]. The more variable incidence of ILC has been attributed to its stronger dependence on female hormone exposure[64].

While IDC and ILC both arise from the TDLU, their pathology differs in several ways. First, ILC tends to have better short and long-term survival than IDC, which is likely due to the former commonly being low-to-middle grade, exhibiting low proliferative markers (Ki-67) but high expression of hormone receptors, and lacking HER2 overexpression or amplification[60,65,66]. However, this may be primarily due to a bias on tumor stage at diagnosis, as a large stage- matched study comparing IDC and ILC demonstrated worse long-term prognosis for the latter[67]. Moreover, due to their lower grade and higher expression of hormone receptors than IDC, ILC tends to be more chemoresistant, though some patients can achieve pathologic complete response[60,68]. Finally, while the rates of metastasis are similar between IDC and ILC, the sites of metastasis vary. For example, ILC tends to metastasize more to the gastrointestinal system, peritoneum, and gynecologic organs, while IDC metastases are found more commonly in the lung, pleura, and bone[69]. In summary, invasive ductal carcinoma and invasive lobular carcinoma are complex diseases with differing prognoses and therapies. For the sake of this dissertation, invasive ductal carcinoma will be the focus, as it is the most common breast cancer in women and also the tumor type of all commonly used human breast cancer cell lines, such as MCF-7, MDA-MB-231, T-47D, and BT-474.

1.5.2 Clinical Classification of Breast Cancer

In addition to classifying tumors based on histological appearance (Section 1.5.1), breast cancers are clinically ranked by tumor stage and grade. Tumor stage refers to the clinical status of the

patient and is determined by the TNM system, initially established by the American Joint Committee on Cancer (AJCC)[70]. TNM is an acronym for the three components that determine the cancer stage: Tumor (T), Nodal status (N), and Metastasis status (M). A specific TNM classification system exists for each type of cancer, so only the specific AJCC criteria for breast cancer are listed in Table 4[71].

Table 4. Breast cancer stages

Stage	Primary Tumor	Nodes	Metastasis
IA	≤ 20mm	None	None
IB	None ≤ 20mm	Nodal micrometastasis (0.2-2mm or >200 cells)	None
IIA	≤ 20mm 20-50mm	N1 None	None
IIB	20-50mm > 50mm	N1 None	None
IIIA	≤ 50mm > 50mm	N2 N1 or N2	None
IIIB	T4	None, N1, or N2	None
IIIC	Any size	N3	None
IV	Any size	Any status	Yes

Breast cancer stages as according to the TNM staging system developed by the AJCC in their 8th edition manual[71]. AxN = Axillary lymph node; IMN = internal mammary lymph node; ScLN: supraclavicular lymph node; IcLN: infraclavicular lymph node; SLNB = sentinel lymph node biopsy; Tumor abbreviations: T4 = any size tumor with extension into the chest wall and or skin (ulceration/nodules); Nodal Abbreviations: N1 = 1-3 AxN/IMN on SLNB; N2 = 4-9 AxN on SLNB or clinically detected IMN; N3 = ≥10 AxN, ≥1 IcLN, clinically detected IMN + ≥1 AxN or IMN + >3 AxN on SLNB, or ≥1 ipsilateral ScLN.

The full breast cancer staging criteria are described in Table 4[71]. The most tangible definitions are as follows. Breast cancer can be classified as Stage 0, I, II, III, or IV. Stage 0 breast

cancer is ductal carcinoma in situ (DCIS), which has not progressed to invasive breast cancer. Stage I breast cancer applies to primary tumors less than 2cm in size and is further separated into IA and IB, with the latter exhibiting nodal micrometastases. Stage II breast cancer is similarly separated into IIA and IIB, with IIB exhibiting either a larger primary tumor or higher nodal status. Stage III breast cancer is subclassified into IIIA, IIIB, and IIIC, with increasing levels of invasion. For example, Stage IIIA cancers have lower nodal involvement than Stage IIIC, while Stage IIIB tumors involve extension to the chest wall or skin. Finally, Stage IV cancer refers to breast cancer with detectable metastases and is not dependent on tumor size or nodal status.

While tumor stage is a clinical diagnosis based on a combination of the tumor size, nodal status, and metastasis status, tumor grade is dependent on cellular morphology and histology determined by cytological analyses. The most common grading system used in the US is the Nottingham Histologic Score[72,73], whose parameters are listed in Table 5.

Table 5. The Nottingham histologic score

Histologic Parameters	**Score**		
	1	2	3
Tubule Formation	> 75%	10-75%	< 10%
Nuclear Pleomorphism	Small, regular, and uniform	Moderate increase in size and variability	High variability
Mitotic Counts / 10 HPF	0-5	6-10	> 10
Total Score	3-5	6-7	8-9
Grade	1 (Low)	2 (Moderate)	3 (High)

Tubule formation, nuclear pleomorphism, and mitotic counts are determined for breast cancer tumor sections[72,73]. Mitotic count cutoffs listed are for a microscope with a field area of 0.152 mm^2 as reported[72]. HPF = high powered field.

Tubule formation refers to the appeared differentiation of the histological sample, namely how glandular (with patent lumens) the tumor cell organization is. Tubules are a sign of well-differentiated tumors, as they resemble the physiological glandular breast. Nuclear pleomorphism refers to the morphology of the nuclei of the tumor cells. A higher degree of variability and larger size of cancer cell nuclei are found in higher grade tumors. Finally, the mitotic count assesses the proliferative capacity of the tumor, with a higher mitotic score corresponding to a higher grade tumor. The Nottingham Histologic Score has been proven to be as effective as nodal status and more effective than primary tumor size at predicting patient prognosis[72,74].

1.5.3 Molecular Classification of Breast Cancer

The molecular classification of breast cancer has become more complex, largely due to genomic advances in the past decade. For the purpose of this dissertation, only the four most common subtypes of breast cancer will be discussed: Luminal A, Luminal B, HER2 positive, and basal-like / triple negative[75,76]. Claudin-low and normal-like breast cancers are newer subclasses of basal-like / triple negative breast cancer, marked by lack of expression of basal markers like cytokeratins 5 and 14[77,78]. For contextual reasons, these two subtypes will be grouped with other triple negative breast cancers. It should be noted that although these four subtypes are deemed to express or lack specific molecular markers, some tumors deviate from these criteria and some individual tumors can contain multiple subtypes[78].

Luminal breast cancer was initially described as a single type of breast cancer based on ER positivity (ER) and gene expression data[79]. Now, it is recognized that two forms of luminal breast cancer exist – luminal A and luminal B – which have different prognoses and tumor traits. Luminal breast cancer accounts for 60-70% of all breast cancer, with approximately 1/3rd of luminal cases

corresponding to the luminal B subtype[76,80,81]. Since all luminal breast cancers are ER positive, the A and B subtypes are distinguished by tumor expression of HER2, Ki-67, and progesterone receptor (PR). Whereas luminal A cancers are mostly HER2 negative, Ki-67 low, and PR high, luminal B can present with either positive or negative HER2 expression. HER2 positive luminal B tumors are ER positive and have no criteria for Ki-67 or PR expression, whereas HER2 negative luminal B tumors are ER positive and either Ki-67 high or PR low[82]. Since luminal B tumors either express HER2 or have high Ki-67, they are more aggressive, and their prognosis is worse than that of luminal A breast cancers[80,82,83].

HER2 type tumors were initially identified for their positivity for the human epidermal growth factor receptor 2 (HER2) and make up approximately 10-15% of breast cancers[75,80,81]. It should be noted that this classification is determined by gene expression clusters and not necessarily protein expression by immunohistochemistry, as 30% of HER2 type tumors do not respond to HER2-targetted therapy and are clinically negative for HER2 enrichment[84]. Approximately 50% of HER2 type tumors also express hormone receptors[85], and HER2 type tumors are clinically more aggressive than luminal type tumors[80,83,86].

Finally, basal-like / triple negative breast cancers account for 15-20% of all breast cancers and are aggressive like HER2 positive disease[80,81,87]. This subtype commonly groups basal-like tumors with triple-negative tumors; however, these are not inclusive entities. Breast cancers that are negative for ER, PR, and HER2 are defined as triple negative[78,88]. Similar to these tumors, those that express one or more high molecular weight cytokeratins present in the normal basal epithelium of the breast (CKs 5, 6, 14, and 17), and commonly also lack ER, PR, and HER2 expression, are referred to as basal-like[78]. As stated briefly above, normal-like and claudin-low are two forms of triple negative breast cancer that are not basal-like due to lack of expression of basal

markers[78]. Half of basal-like tumors express EGFR, which negatively impacts survival,independent of tumor size or nodal status[87,89].

In summary, the four main molecular classifications of breast cancer can be defined by expression of markers and dictates disease prognosis. Patients with luminal A disease have a fairly good prognosis, patients with luminal B have an intermediate prognosis, and patients with HER2-type or basal-like / triple negative breast cancers have poor prognosis[75]. The molecular characteristics of each of these subtypes are shown below in Table 6. Careful classification of breast cancers is critical to identifying the signatures of tumors that will respond best to a particular therapeutic approach.

Table 6. Molecular markers for cancer subtypes

Subtype	ER	PR	HER2	Ki-67	Basal CK
Luminal A	+	+	-	-	-
Luminal B	+	- / +	+	- / +	-
	+	+	-	+	-
	+	-	-	-	-
HER2-Type	- / +	- / +	+		
TNBC	-	-	-	- / +	- / +
Basal-like	-	-	-	- / +	+

Luminal A, Luminal B, HER2-type, triple negative, and basal-like breast cancer gene expression patterns. TNBC = Triple Negative Breast Cancer; ER = estrogen receptor, PR = progesterone receptor; CK = cytokeratin.

1.6 CELLULAR AND MOLECULAR PATHOLOGY

The progression of a particular instance of breast cancer depends not only on its classification according to the three systems discussed above, but also the behavior of the relevant signaling pathways and genes that are often implicated in breast carcinogenesis and progression. In particular, the hormone receptors (estrogen receptor and progesterone receptor), the ErbB family proteins, the mitogen activated protein kinase (MAPK) cascade, and the phosphoinositide 3-kinase – Akt signaling axis are the most relevant to breast cancer pathogenesis. It is important to acknowledge that the signaling networks presented below are simplified for clarity. In the past decade, breast cancers have been grouped more sophisticatedly by analyzing RNA expression and micro-RNA type and abundance. For a more extensive review of these items and other relevant markers in breast cancer, the reader is directed to more comprehensive references[90–99].

1.6.1 The Estrogen Receptor

1.6.1.1 History

The estrogen receptor has been implicated in the pathogenesis of breast cancer since Dr. George Beatson induced regression of metastatic breast cancer by surgical removal of both ovaries (bilateral oophorectomy) in 1896[100]. The estrogen receptor was first described by Jensen in 1958 when he noticed the accumulation of radiolabeled estrogen in estrogen-target tissues, such as the uterus[101]. Other scientists noticed similar findings in both the female reproductive organs and breast cancers[102,103]. Starting in the 1970s, the biology of the estrogen receptor in the context of cancer had been determined, and its overexpression was hypothesized as a predictive factor for responsiveness to hormone therapy (Section 1.10)[104–107]. In 1974, comprehensive data were

presented in the support of using estrogen receptor expression as a predictive marker for response to endocrine therapy in breast cancer and to suggest tumors without its expression will not respond to endocrine therapy[108,109]. Moreover, lack of estrogen receptor expression was found to be an independent prognostic factor for early cancer recurrence[110].

Detection methods for estrogen receptor expression on tumor tissue evolved over the following several decades. Initial tests to determine the expression of estrogen receptor consisted of homogenization and purification of tumor lysate from fresh frozen tissue and assessment of ligand (estradiol) binding[106,109]. Separation of bound and free estradiol was most commonly achieved by centrifugation with dextran-coated charcoal[106,111], although this technique may be inaccurate for dilute tumor lysate samples[112]. Advancements in the detection of estrogen receptor expression were made with the discovery of specific monoclonal antibodies in 1984 that suggested primarily nuclear localization of the estrogen receptor[113]. These antibodies were first used for an estrogen receptor monoclonal enzyme immunoassay (ER-EIA) that demonstrated higher sensitivity and specificity for proper classification of tumor specimens[114]. Moreover, tumor samples did not need to be processed immediately, as ER-EIA could be performed on 6-month old cryopreserved samples[114]. In the 1990s, immunohistochemical (IHC) determination of estrogen receptor in frozen and paraffin embedded sections was developed, with higher accuracy initially obtained using the former[115]. By the early-to-mid 1990s, IHC determination of estrogen receptor status became the standard due to convenience, low-cost, and high sensitivity[116,117]. Clinical studies have classified the relative abundance of ER required for disease free survival and overall survival benefits with hormone therapy[118].

1.6.1.2 Molecular Biology

The estrogen receptor is a nuclear hormone receptor with two isoforms: ERα and ERβ. The estrogen receptor (later determined to be ERα) was first discovered by Jensen in 1958. The full ERα, encoded by the ESR1 gene, was first successfully cloned from MCF-7 breast cancer cells in 1985 by Walter and colleagues[119]. A decade later, a second isoform of the estrogen receptor, ERβ, encoded by the ESR2 gene, was described and cloned in 1996 first by Kuiper and subsequently by Mosselman[120,121]. The specific role of ERβ remains unclear. Some studies suggest ERβ expression correlates with improved overall survival, possibly through suppression of cell proliferation.[122,123] However, currently used ERβ antibodies are non-specific, which precludes any concrete conclusions from being drawn[124]. As such, for the remainder of this section, ERα will be discussed exclusively.

The estrogen receptor can influence gene transcription through many different mechanisms. First, the estrogen receptor can act in the nucleus to influence gene transcription by either direct binding to the DNA at estrogen response elements (EREs) or by association with other transcription factors such as Sp-1, AP-1, and NF-κB[95,96,125]. Second, the estrogen receptor can act in the mitochondria to inhibit the early stages of apoptosis through transcription and augmentation of signaling through factors such as NRF-1[96,126,127]. Finally, and especially pertinent for breast cancer, estradiol binding allows the estrogen receptor to interact with signaling pathways such as MAPK and PI3K-Akt[128–130]. In summary, the estrogen receptor has multiple downstream pathways through which it can signal, both through direct interactions with transcriptional machinery and through interacting with other signaling networks. For a more thorough review of the mechanisms of action of the estrogen receptor, the reader is directed to the literature[95,96,131,132].

The estrogen receptor plays both physiological and pathological roles in the human breast. In normal physiology, ERα is essential for mammary gland morphogenesis and ductal elongation. Importantly, ERα expression is critical both in the mammary epithelial cells and in the stroma. For example, transplantation of a receptor negative fat pad into wild type mice is sufficient to inhibit ductal ingrowth due to a requirement for growth factor secretion by ERα positive stromal cells[133]. In breast cancer, estrogen receptor overexpression is the most common mechanism for pathology, almost half of which can be attributed to gene amplification[134]. Constitutively active mutations in ERα have been described[135]; however, only 1% of primary breast cancers demonstrate mutations in the coding region of ERα[136], so these mutations are not relevant to most breast cancer cases. Additionally, it should be noted that ERα overexpression can also induce ligand-independent signaling[137]. Other mechanisms for ERα upregulation in breast cancer include altered promoter activity and decreased protein degradation[138,139].

In summary, the estrogen receptor has played the largest role in understanding breast cancer pathogenesis and responsiveness to therapy. The estrogen receptor isoform alpha (ERα) acts through transcription (direct or indirect through other transcription factors) and through interactions with other signaling factors (PI3K and tyrosine kinases involved in the MAPK cascade) to induce proliferation of breast epithelial cells during physiological development and malignancy. In breast cancers, half of estrogen receptor positive cancers are due to gene amplification of ESR1, whereas the other half are due to altered promoter activity or decreased protein degradation. Estrogen receptor positivity is an important prognostic factor and predictive factor for response to hormone therapies (Section 1.10). The interactions between the estrogen receptor and other oncogenes implicated in breast cancer, such as EGFR and HER2, are important for optimization of therapy for patients with estrogen receptor positive breast cancer[140,141].

1.6.2 The Progesterone Receptor

The progesterone receptors A and B (PR-A and PR-B) have been significantly less studied in breast cancer than the estrogen receptor, yet they play significant roles in both mammary development and cancer pathogenesis. Currently, there are no FDA approved anti-progesterone therapies for breast cancer, as agents tried in clinical trials present with severe side effects or cross-react with the glucocorticoid receptor[142]. Longitudinal clinical studies have in fact demonstrated an increased incidence of breast cancer in women receiving combined (estrogen + progestin) hormone therapy as compared to estrogen alone[143]. Therefore, information on the progesterone receptor presented below is focused on its role in physiology, its molecular signaling networks, and its use as a prognostic marker in breast cancer, rather than any future therapeutic modality.

The progesterone receptor plays several important roles in normal breast physiology. Progesterone receptor stimulation is responsible for alveologenesis and alveolar organization into lobules during puberty and also in lobular-alveolar expansion during pregnancy in preparation for lactation[144,145]. Moreover, the high levels of progesterone during the luteal phase of the menstrual cycle correlate with a high proliferative index of the breast epithelium[144]. Of the two progesterone receptor isoforms, PR-B plays the most significant role in breast epithelial cell proliferation, as knock out of PR-A in mice results in a breast glandular structure similar to that of wild type mice[146]. Finally, progesterone action suppresses milk protein synthesis, which is particularly important in the late stages of pregnancy[144]. For a complete review of the role of progesterone receptor in normal physiology, the reader is referred to the following comprehensive reference[144].

Progesterone receptor (PR) is commonly implicated in breast cancer, with two-thirds (66%) of breast cancer patients classifying as PR positive[81]. Importantly, the progesterone receptor is directly regulated by ERα, and as such is often clinically used as an indicator for estrogen

receptor activity[147]. While the progesterone receptor isoforms (PR-A and PR-B) are expressed 1:1 in normal tissue, this ratio is often altered in breast cancer, predominantly with a decrease in PR-B[93,148,149]. While the relative loss of PR-B could be explained by an overexpression of PR-A, the majority of the data is in support of increased phosphorylation-turnover of PR-B, reflective of a high degree of crosstalk between PR and other signaling axes such as MAPK[149–151] and the estrogen receptor[152]. As proliferative signaling in the breast is primarily due to PR-B activity and a higher relative expression of PR-A is correlated with higher tumor grade[153], it is likely that increased signaling from PR-B causing increased turnover results in an apparent loss of PR-B despite its heightened activity[145,149]. Clinical detection of PR by IHC is not isoform specific. As such, altered ratios between PR-A and PR-B may explain some of the variability in responsiveness of PR positive tumors to endocrine therapy[152].

Expression of the progesterone receptor is a useful prognostic marker for breast cancer, and is predictive of increased responsiveness to endocrine therapy and decreased recurrence[147,154–156]. Since progesterone receptor is directly regulated by estrogen receptor, tumor cells addicted to estrogen receptor signaling, and thus responsive to hormone therapy, are likely to express higher levels of progesterone receptor[147]. More recent studies have suggested that lack of progesterone receptor expression, independent of estrogen receptor status, is a negative prognostic marker for overall survival, breast cancer-specific survival, and disease-free survival[157].

In summary, while traditionally used as an indicator of estrogen receptor activity, the progesterone receptor serves multiple roles in the physiological and pathological human breast. Progesterone signaling is important for breast development during puberty and pregnancy and also plays critical roles in timing lactogenesis. In breast cancer, progesterone receptor isoform expression is altered, with the observed loss of progesterone receptor and corresponding poor

clinical prognosis likely due to increased signaling and degradation of PR-B. While estrogen receptor directly regulates progesterone receptor levels, PR is an independent beneficial prognostic marker for breast cancer, as patients harnessing tumors with absent PR expression have worse overall survival. These findings dictate the importance of progesterone receptor in the pathogenesis and prognosis of breast cancer.

1.6.3 The ErbB Receptor Family

The ErbB family of proteins contains four tyrosine kinase receptors all structurally related to the epidermal growth factor receptor (EGFR). The four receptors are ErbB1 (EGFR), ErbB2 (HER2/Neu), ErbB3 (HER3), and ErbB4 (HER4). As EGFR and HER2 are the most commonly implicated and targeted for therapy in breast cancer, these two isoforms will be the primary focus of this section, whereas HER3 and HER4 will be discussed in less detail.

1.6.3.1 The Epidermal Growth Factor Receptor (EGFR)

The epidermal growth factor receptor (EGFR), also known as HER (Human EGF Receptor), is present on all epithelial and stromal cells, and its expression is polarized to the basolateral surface to prevent autocrine signaling from luminal EGF[158]. Approximately 18-24% of breast cancers overexpress EGFR, and roughly half of these are due to an increase in gene copy number[159–161]. EGFR expression is even more common in triple negative breast cancer, and its expression is inversely correlated with hormone receptor expression[162,163]. EGFR serves as an upstream regulator of several signaling cascades. The Ras – mitogen activated protein kinase pathway (Ras – MAPK) and the phosphatidyl inositol 3-kinase and Akt pathway (PI3K – Akt) are the most significant to breast cancer[164] and are discussed further in the Sections 1.6.4 and 1.6.5.

EGFR signaling is complex and only the essential features will be described here. For more details, the reader is referred to the literature[158,165]. In short, non-ligand bound EGFR exists in a preferentially closed conformation, preventing association with like receptors. Once EGFR binds to a ligand, its open conformation is stabilized which allows for homo- and hetero-dimerization with EGFR or other members of the ErbB family respectively[166]. EGFR has seven known ligands: epidermal growth factor (EGF), betacellulin, heparin-binding EGF (HB-EGF), transforming growth factor alpha (TGFα), amphiregulin, epigen, and epiregulin, with the last three exhibiting approximately 10-100 fold lower affinity for EGFR than the first four[167]. EGFR dimerization facilitates activation of the cytoplasmic kinase domain and subsequent cross-phosphorylation of the c-terminal receptor tails. Phosphorylation allows for the recruitment of proteins harboring Src homology 2 (SH2) and phosphotyrosine-binding (PTB) domains, which subsequently instigate downstream intracellular signaling[167]. After activation of EGFR, the receptor complex is internalized and either recycled back to the plasma membrane or degraded in the lysosome. The fate of internalized EGFR is dependent on the ligand to which it is bound, with higher affinity and non-dissociable ligands (such as EGF) preferentially driving degradation[158].

EGFR plays dynamic roles in the progression of breast cancer. Clinically, EGFR over-expression is a poor prognostic indicator in breast cancer[89,159] because it has contrasting effects on tumor biology at the site of the primary tumor and at distant metastatic lesions. At the site of the primary tumor, EGFR plays a significant role in breast cancer EMT, often through autocrine EGFR activity that drives E-cadherin internalization[45]. Moreover, autocrine EGFR signaling stimulates tumor cell proliferation and invasion[45]. In contrast, progressing macrometastases exhibit decreased expression and nuclear localization of EGFR, which induces resistance to EGFR-targeted therapies. Moreover, EGF-stimulation can result in growth arrest and apoptosis of tumor cells at

the metastatic site[165]. That EGFR plays significant roles across the entire breast cancer metastatic cascade demonstrates its importance in human breast cancer.

1.6.3.2 The Human Epidermal Growth Factor Receptor 2 (HER2)

HER2 gene amplification, leading to its overexpression at the protein level, was first demonstrated in breast cancer by Slamon and colleagues in 1987[168]. This study linked overexpression of HER2 to decreased overall survival and decreased relapse-free survival. The human epidermal growth factor receptor 2 (HER2) is overexpressed in 15-30% of human breast cancers and remains a marker of more aggressive disease despite significant advances in HER2 targeted therapies[169–171]. In contrast to the other three members of the ErbB receptor family (EGFR, HER3, and HER4), HER2 has no known ligands. It homo- or hetero-dimerizes with other members of the HER family to influence downstream signaling in a manner similar to that discussed in the "EGFR" section previously[170], with the involvement of signaling pathways such as Ras – MAPK and PI3K – Akt.

While HER2 can form both homo- and hetero-dimers with other members of the HER family, the latter more strongly stimulates mitogenic signaling[169]. Homodimers in human tumors are thought to be due to a high receptor density at the cell membrane, allowing spontaneous dimerization[172]. Indeed, expression of a high membrane density of HER2 in human cells is sufficient for oncogenic transformation[173]. Analysis of the HER2 analog in rodents, neu, has also demonstrated that point mutagenesis at key residues can enhance homodimerization, although human tumors do not demonstrate the same mutation[172]. HER2 is the preferential partner for EGFR, HER3, and HER4 for heterodimerization[174], with HER2-HER3 heterodimers being most preferable. This finding is also illustrated by the relative mitogenic potentials of different homo- and hetero-dimers, shown in Table 7[169].

Table 7. HER family dimer mitogenic potential

Ligand bound:	EGFR	HER2	HER3
EGFR	3.2	N/A	6.5
HER2	5.0	3.1	10.5
HER3	3.1	N/A	0

The relative mitogenic potential of different homo- and hetero-dimers of the HER family. The top row illustrates the member of the HER family that is ligand bound (in the case of hetero-dimers). HER2 homodimer mitogenic potential was demonstrated by dimerization with a non-blocking antibody. HER4 data are not included as this receptor antagonizes cell proliferation. Data are modified from Yarden et al, 2001[169].

Hetero-dimers signal more potently than their homodimer counterparts, primarily due to lower rates of ligand dissociation, which is further enhanced when HER2 is the binding partner[175,176]. Prolonged binding allows for preferential mitogenic (sustained) signaling through mediators such as the MAPK pathway[169]. In addition to reducing ligand dissociation rates, HER2 can prolong mitogenic signals by enhancing receptor recycling upon ligand-stimulated internalization of dimers. For example, while EGFR homodimers that bind high affinity ligands are targeted for degradation by adapter proteins (such as the ubiquitin ligase c-Cbl), EGFR-HER2 hetero-dimers bind these adapter proteins with less affinity, facilitating recycling of EGFR to the membrane and prolonged signaling. Interestingly, one of the proposed mechanisms of Herceptin (anti-HER2 monoclonal antibody discussed in more detail in Section 1.10) is recruitment of c-Cbl to HER2 molecules, facilitating receptor degradation[169,177].

In summary, HER2 acts as an oncogenic driver in breast cancer through gene amplification and protein over-expression. Most commonly, HER2 associates with EGFR, HER3, and HER4 in hetero-dimers to potentiate and prolong mitogenic signaling and decrease ligand-facilitated internalization and degradation of signaling complexes. HER2 can also spontaneously

homodimerize at high membrane receptor densities and directly signal to downstream pathways such as Ras – MAPK and PI3K – Akt. HER2 is an important therapeutic target in breast cancer and its overexpression is predictive of response to anti-HER2 based therapies. Targeted therapies against HER2 will be discussed in detail in Section 1.10.

1.6.3.3 The Human Epidermal Growth Factor Receptor 3 (HER3)

HER3 is uniquely different from the other three HER family members in that the alteration of several critical residues in the kinase domain inactivates its catalytic function[178]. Thus, while heterodimers containing HER3 are capable of downstream signaling, HER3 homodimers cannot signal because they lack the ability to phosphorylate the C-terminal residues that serve as signaling protein docking sites[178]. Moreover, heterodimers containing ligand-bound HER3 are capable of more prolonged mitogenic signaling than other receptor pairs[169], in particular in HER2 amplified breast cancer[179]. HER3 has two known ligands, Neuregulin-1 and -2 (NRG1, NRG2), which mediate homo- and hetero-dimerization.

HER3 contains multiple docking sites for p85 PI3K, which can mediate resistance to anti-HER2 therapy in amplified tumors[179] and contribute to metastatic colonization[180]. While suppression of EGFR or HER2 signaling using tyrosine kinase inhibitors transiently suppresses HER3 phosphorylation, an upregulation of HER3 protein at the membrane and reduced phosphatase activity eventually causes restoration of oncogenic signaling[181]. This resistance signaling is mediated by a suppression of PI3K/Akt signaling that induces transcription factors, such as FoxO1, that drive upregulation and phosphorylation of multiple receptor tyrosine kinases including HER3[182]. Thus, HER3 acts as a significant mediator of anti-HER therapy resistance and oncogenic signaling in breast cancer. While these data suggest that HER3 would be a poor

prognostic marker in breast cancer, clinical studies remain inconclusive on its influence on breast cancer survival[183].

1.6.3.4 The Human Epidermal Growth Factor Receptor 4 (HER4)

In contrast to the other three HER family members, HER4 signaling is associated with anti-proliferative and pro-apoptotic effects in breast cancer[183]. HER4 can bind to many ligands, including all of the Neuregulin growth factors (NRG-1, -2, -3, and -4), betacellulin, epiregulin, and HB-EGF[183,184]. Nuclear localization of HER4 results in anti-proliferative effects in breast cancer cells, while mitochondrial localization of HER4 and subsequent interaction with BAK promotes apoptosis[184]. As expected, HER4 expression in breast cancer is associated with lower grade, hormone receptor positive disease[185]. Moreover, HER4 expression is an independent prognostic marker for improved overall survival in breast cancer[185–187]. Finally, HER4 has been shown to antagonize HER2 signaling both *in vitro* and in clinical studies[187].

1.6.3.5 ErbB Family Summary

In summary, the ErbB / HER family plays significant pathologic and prognostic roles in breast cancer. EGFR is associated with tumor invasion and metastasis due to its role in promoting EMT. HER2 is associated with oncogenic transformation and proliferation of breast cancer cells and is the most common target of antibody-based therapy in breast cancer. HER3, while kinase inactive, contributes to mitogenic signaling and anti-HER therapy resistance in breast cancer. Finally, HER4 signals for growth inhibition and apoptosis of breast cancer cells, and its expression bears good clinical prognosis. The effects of these four receptors are summarized in Table 8.

Table 8. HER family effects in breast cancer

Effects in Breast Cancer	EGFR	HER2	HER3	HER4
Oncogenesis and Growth	+/-	+	+	-
Invasion and Metastasis	+	+	+	-
Clinical Prognosis	-	-	+/-	+

The influences of the HER family members on oncogenesis and growth, invasion and metastasis, and clinical prognosis of breast cancer. (+) positive effector, (-) negative effector, (+/-) context-dependent effector.

1.6.4 Ras – MAPK Signaling

The Ras – MAPK signaling cascade plays important roles in the progression of breast cancer. This is not meant to be a comprehensive review, rather to provide background sufficient to understand data presented herein. There are four major mitogen-associated protein kinase (MAPK) signaling cascades in humans, all of which end with transcriptional activity performed by the association of the MAP kinase in the pathway with nuclear transcriptional factors. These four effector MAP kinases are Erk1/2, Erk5, Jnk, and p38[188]. Generally, Erk1/2 and Erk5 are responses to extracellular growth factor stimulation, whereas Jnk and p38 are associated with cellular stress signals[188–190]. As the present work only involves Erk1/2, it is only necessary to discuss this MAPK effector and its upstream factors, focusing on the signaling cascade followed by the *in vitro* and *in vivo* influences of this signaling cascade on breast cancer progression. Finally, the clinical relevance of this pathway is described.

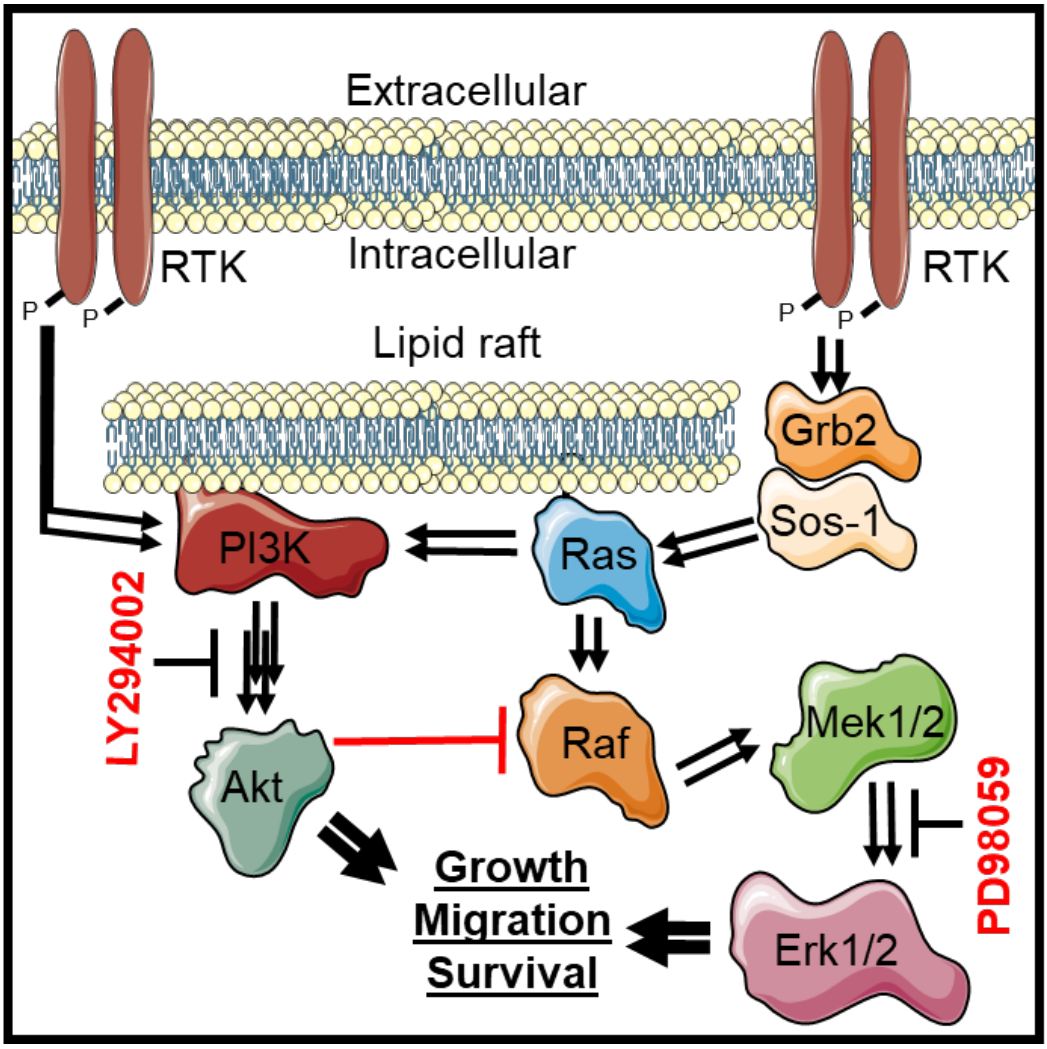


Figure 6. The Ras - MAPK and PI3K - Akt signaling pathways

A simplified representation of the Ras – MAPK and PI3K – Akt signaling networks, illustrating the crosstalk between the pathways using a receptor tyrosine kinase (RTK) common input. Phosphorylation of the intracellular domain of RTK (for example, EGFR) attracts the SH2 domain of Grb2, which subsequently attracts Sos-1 through its SH3 domain. Sos-1 acts as a guanine exchange factor (GEF) for Ras, converting it into the "on" state. Ras signals downstream through Raf and PI3K as its major effectors. PI3K signals through Akt whereas Raf signals through Mek1/2, then Erk1/2, to activate transcriptional programs that lead to growth migration and survival. RTKs can also directly signal through PI3K. Phosphorylated Akt can suppress Raf activity as one of the mechanisms of crosstalk between these two signaling networks. Inhibitors of PI3K (LY294002) and Mek1/2 (PD98059) signaling are illustrated. This diagram was created using images from Servier Medical Art[46].

Since Erk1/2 signaling commonly begins with growth factors binding to the extracellular domain of receptor tyrosine kinases (RTKs), EGFR activation will be used to illustrate this signaling pathway (Figure 6). EGFR binds ligand, which induces receptor homo- or hetero-dimerization and phosphorylation of the cytoplasmic receptor tails[158]. Phosphorylation of the EGFR intracellular domain recruits proteins harboring SH2 and PTB binding domains, such as Grb2[191,192]. In turn, Grb2 associates through its SH3 domain with the guanine exchange factor (GEF) Sos-1[193,194]. Sos-1 catalyzes the exchange of GDP for GTP on several small signaling G-proteins, such as Ras, which acts as an activation signal. Importantly, in order to associate with Sos-1, Ras must be tethered to either the plasma membrane or lipid rafts through prenyl groups[195].

After activation, Ras recruits Raf to the membrane through the latter's Ras binding domain (RBD), which removes Raf auto-inhibition and allows for partial activation of Raf kinase activity by its phosphorylation by other membrane kinases, such as CK2 and Shc[196,197]. Dimerization of Raf induces its catalytic activity and results in the recruitment of Mek1/2 and its phosphorylation and activation[197]. Activated Mek1/2 subsequently phosphorylates and activates Erk1/2, which translocates to the nucleus and interacts with transcription factors such as Elk1 and Elf1 to induce transcription of target genes[197,198]. Importantly for breast cancer, nuclear Erk1/2 can interact with and activate the estrogen receptor[198]. Finally, feedback inhibition of this MAPK cascade acts as an important regulatory mechanism whereby, for example, active Erk1/2 phosphorylates upstream proteins at inhibitory sites to reduce downstream activation of the pathway[199].

Erk1/2 signaling in breast cancer can be described as either independent of or dependent on estrogen signaling. Estrogen independent Erk1/2 activity is traditionally due to upstream signals from growth factors, such as EGF, TGFα, and IGF-1, which transmit mitogenic and migratory signals down the MAPK cascade[200]. In contrast, estrogen dependent Erk1/2 activity can occur from

both immediate (signal-based) and late (genomic) effects. Estrogen can activate Erk1/2 within five minutes of stimulation in ER positive MCF-7 breast cancer cells, which is due in part to activation of c-Src[201]. Similarly, the estrogen receptor mediates transcription of growth factors such as TGFα and IGF-1, which can subsequently act in an autocrine or paracrine manner to enhance Erk1/2 activity[202]. Importantly, enhanced Erk1/2 activity is commonly found in breast cancers subjected to anti-estrogen therapies, suggesting this pathway serves as an adaptation mechanism. For example, breast cancer xenografts formed from long-term estrogen deprived cells were more sensitive to the mitogenic effects of estrogen than their wild type counterparts, which was due to increased MAPK activity in the former[203]. Moreover, breast cancer cells resistant to anti-estrogen therapies rely in part on Erk1/2 activation for cell growth[204].

Hyper-activation of Erk1/2 is common in breast cancer pathologic specimens. For example, there is a 5-fold upregulation in both phosphorylation and protein expression of Erk1/2 in breast carcinoma as compared to benign pathologies, such as fibroadenoma[205], and similar upregulation compared to matched control tissue[206,207]. Likewise, Erk1/2 activity is further increased in lymph node metastases as compared to primary breast tumors, suggesting it plays an important role in the metastatic cascade[207]. Moreover, highly activated MAPK signatures are associated with loss of estrogen receptor expression in clinical breast cancer specimens, decreased responsiveness to anti-estrogen therapy, and poor prognosis[208–210].

In summary, the Ras – MAPK signaling pathway, focusing specifically on Erk1/2 as the downstream effector, is commonly dysregulated in breast cancer. Mitogenic and migratory signals from Erk1/2 originate from growth factor stimulation, through EGFR and other RTKs, and from estrogen stimulation, through the estrogen receptor and c-Src. Enhanced Erk1/2 activity in breast

cancer is a resistance mechanism to anti-estrogen therapy, is implicated in tumor metastasis, and bears poor clinical prognosis.

1.6.5 PI3K – Akt Signaling

Another important factor in breast cancer is the PI3K – Akt signaling cascade, particularly PI3K activation, recruitment of Akt, and downstream signals. The *in vitro* and *in vivo* influences of this signaling cascade on breast cancer progression will be discussed and then the clinical relevance of this pathway will be examined.

The phosphatidylinositol 3-kinase (PI3K) family of enzymes comprises many different classes and subclasses based on enzyme structure and activity. Generally, PI3K is composed of p110 (catalytic, PI3KCA gene) and p85 (regulatory) subunits[211]. Activation of PI3K can occur through at least three independent pathways, all of which involve binding of ligand and activation of RTKs[212]. First, the SH2 domain of p85 can directly bind phospho-tyrosine consensus sequences on the cytoplasmic tails of activated RTKs, which in turn activates the p110 catalytic subunit[213]. Second, Grb2 association with the RTK can recruit Gab, which in turn can bind p85 and activate p110[214]. Finally, Ras can directly activate p110, independent of p85 involvement[212] (Figure 6).

Downstream signaling is initiated by the kinase activity of activated p110, which generates the second messenger phosphatidylinositol 3,4,5-triphosphate (PIP_3) by phosphorylation of its precursor, phosphatidylinositol 4,5-bisphosphate (PIP_2)[215]. Subsequently, PIP_3 can initiate intracellular signaling by binding to Pleckstrin Homology (PH) domains of downstream kinases, such as phosphoinositide-dependent kinase 1 (PDK1) and Akt[212,216]. The ensuing recruitment of both PDK1 and Akt to the membrane by PIP3 binding brings these two proteins into close proximity and allows the former to activate the latter by phosphorylation at threonine 308[217]. A

second phosphorylation at serine 473 is required for the complete activation of Akt, which is predominantly achieved by mTORC2 activity[218,219].

Akt acts through different downstream mediators to promote cell growth and survival. For example, inactivating phosphorylation of pro-apoptotic factors Bad and procaspase-9 and negative regulation of NF-κB promote cellular survival[212,220]. Moreover, inhibition of GSK3 by Akt prevents the phosphorylation of cytoplasmic β-catenin and its subsequent degradation, which allows β-catenin to translocate to the nucleus and induce the expression of growth promoting genes such as Cyclin D1[220]. Finally, mTORC1 activation by Akt enhances protein synthesis and regulates ribosomal biogenesis[221]. PI3K – Akt signaling is negatively regulated by the phosphatases PTEN and PP2A, which dephosphorylate PIP3 and Akt respectively[220,222].

In breast cancer specifically, hyper-activation of the PI3K – Akt pathway plays a role in tumor development, progression, and resistance to therapy[223]. While homozygous germline knockouts of PTEN are lethal, heterozygous or mammary gland specific knockouts of PTEN were sufficient to induce spontaneous tumors in mice[224]. Similarly, homozygous and heterozygous knockout of Akt1 prevented or delayed tumor formation in mice that over-expressed mammary gland specific HER2[225]. Additionally, conditional over-expression of PI3KCA or Akt1 in mice was sufficient to induce mammary tumor formation and progression[226,227]. Importantly, Akt isoforms play differential roles in breast cancer development and progression. While Akt1 over-expression strongly induces primary tumor growth, down-regulation of Akt1 with up-regulation of Akt2 induces invasion and metastasis[228]. Finally, activation of the PI3K – Akt pathway is sufficient to induce resistance to endocrine and HER2 directed therapy in breast cancer cell lines *in vitro*, and inhibition of this pathway re-sensitizes cells to therapy[229–231].

Dysregulation of the PI3K – Akt pathway is common in breast cancer. Mutations in PI3KCA are the most common genetic alteration in this pathway, present in approximately 36% of breast cancers,[232] with 80% of these mutations occurring in either the helical or kinase domains[233]. In contrast, only 2% and 3% of breast cancers harbor mutations in Akt and PTEN, respectively[232]. Similar to increased activation of Erk1/2, PI3K-Akt signaling contributes to resistance to endocrine[233,234] and HER2[235] directed therapies. While therapies targeting downstream elements of this pathway (Everolimus to inhibit mTORC1) are clinically approved for breast cancer, PI3K and Akt inhibitors have shown limited success in clinical trials due to off-target toxicities[236–238]. Akt activation in breast cancer bears poor clinical prognosis[228,239].

In summary, PI3K – Akt signaling plays multiple roles in the development, progression, and treatment of breast cancer. PI3K activity is regulated by both membrane (e.g. RTKs) and intracellular (e.g. Ras) kinases. Activation of the PI3K – Akt signaling axis promotes cell cycle progression, enhances protein synthesis, and induces resistance to apoptotic stimuli. In breast cancer, more than one third of tumors harbor an activating mutation in PI3K, which promotes initial tumor growth, invasion and metastasis, and resistance to endocrine and HER2 targeted therapies. Finally, high activity of the PI3K - Akt signaling pathway is a negative prognostic factor in breast cancer.

1.6.6 Ras – MAPK and PI3K – Akt Crosstalk

Crosstalk between the Ras – MAPK and PI3K – Akt cascades has already been introduced in the previous sections. The mechanisms by which the two pathways communicate will be briefly summarized here. First, active (GTP-bound) Ras can directly bind and activate the p110 subunit PI3K[212]. Second, Akt can negatively regulate the MAPK pathway at the level of Raf by inhibitory

phosphorylation[240]. In contrast, Raf – MAPK activity can potently induce Ras and PI3K by autocrine production of growth factors that signal through cell surface RTKs[241]. Third, active Erk1/2 phosphorylation of Gab1 inhibits Gab1-mediated recruitment of PI3K to the membrane, preventing its subsequent activation[214]. In contrast, Erk1/2 phosphorylation of TSC2, a negative regulator of mTORC1 which is also regulated by Akt, promotes activation of mTORC1 in a PI3K-independent manner[242]. Finally, PI3K association with Gab1 in a positive feedback loop can prolong Ras signaling by increasing the number of Grb2 – Sos-1 complexes recruited to the plasma membrane[243]. In summary, the Ras – MAPK and PI3K – Akt pathways are intimately involved with each other, and each can provide both inhibitory and stimulatory regulation of the other (Figure 6).

1.6.7 Tumor Heterogeneity

For four decades, it has been shown that some breast cancers exhibit tumors and metastases with molecular and cellular heterogeneity[244]. While still not completely understood, tumor heterogeneity was initially thought to arise from the clonal evolution of cancer[245]. In this model, tumor cell evolution is stochastic according to Darwinian Evolution, whereby tumor clones with a greater survival or growth advantage emerge[246]. These advantages can be driven by epigenetic changes and mutations, which alter the activity of oncogenes and tumor suppressor genes[247]. More recently, the theory of tumors being driven by cancer stem cells has provided an alternative and complementary explanation for heterogeneity[248]. In contrast to the clonal evolution model, the cancer stem cell model states that heterogeneity is driven by the composition of a small fraction of tumor cells (the stem cells), which creates a hierarchical organization of different, “terminally differentiated” tumor clones[249]. In this way, proliferation and terminal differentiation of a small

subpopulation of stem cells, each of which may exhibit different gene expression profiles, sustains the tumor.

To illustrate the difference between these two theories, let us consider a breast cancer tumor with five major clones being challenged by treatment with a chemotherapy. In the case of the clonal evolution model, the breast cancer subpopulation most resistant to the chemotherapy will selectively outgrow, which may eliminate some of the initial clones and allow for subsequent evolution to occur. In contrast, the cancer stem cell theory suggests that all of the terminally differentiated cancer cells will be eliminated by the chemotherapy while the slowly dividing cancer stem cells will remain, resulting in an eventual repopulation of the initial heterogeneity after removal of the chemotherapy.

Tumor heterogeneity is a major cause of therapy failure and tumor progression. In breast cancer, heterogeneity may present as differential expression of biomarkers (e.g. The Estrogen Receptor) in a single tumor, differences in biomarker expression between primary and metastatic lesions, and differences in tumor cell phenotype or gene expression[250]. In particular, heterogeneity is emphasized in tumors that exhibit high genomic instability[251]. As such, heterogeneity motivates a combination-based approach to treating breast cancer, since a single agent may not be effective against all tumor cells.

1.7 SURGERY

The first recorded mention of breast cancer dates back to the Edwin Smith Surgical Papyrus in 3000-2500 B.C., in which this incurable disease was described as “cool to touch, bulging and spread all over the breast”[252]. Until the late 19th century, treatment of breast cancer consisted of

barbaric surgeries and faith-based healing[252]. The modernization of breast cancer surgery began with Dr. William S. Halstead who performed the first radical mastectomy at Johns Hopkins in June 1889, with particular attention paid to removal of surrounding involved muscle tissue (pectoralis major)[252,253].

Surgical management at the start of the 20th century incorporated the radical mastectomies of Dr. Halstead and the aggressive bilateral oophorectomies and adrenalectomies pioneered by Dr. George Beatson at the end of the 19th century[252,254]. The first major step towards breast-conserving surgery began with a report from Drs. Patey and Dyson in 1948 that demonstrated no difference in survival when the pectoralis major was preserved during mastectomy[255,256]. Further modifications to the radical mastectomy were made by Drs. Auchincloss and Madden, who additionally preserved the pectoralis minor muscle and argued against the paradigm of complete axillary lymph node dissection[256,257].

The first reports of successful breast-conserving therapy (lumpectomy) for early stage breast cancer were published by Veronesi and colleagues in 1981 and demonstrated no difference in recurrence or survival with their quandrantectomy procedure as compared to radical mastectomy[258]. Furthermore, no difference in either overall or breast cancer-specific survival were noticed after twenty years of follow up[259]. Fisher and colleagues demonstrated similar data with a larger cohort of patients, in which survival was equivalent between lumpectomy and total mastectomy[260,261]. The cosmetic outcome of lumpectomy was improved through pioneering less invasive procedures, such as skin- and nipple-sparing mastectomies[262,263]. Further cosmesis can be achieved using breast reconstructive procedures, in which the removed breast tissue is replaced by implants or autologous tissue, in the form of a free flap, perforator flap, or fat graft[264].

Current recommendations for breast cancer surgical management can be classified into three broad categories depending on the stage of the tumor: early stage primary breast cancer, locally advanced primary breast cancer, and metastatic breast cancer. Early stage breast cancers (Stage 0, I, or II) are preferentially treated with breast-conserving surgery. A total mastectomy is used if negative tumor margins cannot be adequately achieved with lumpectomy or if the patient desires a different cosmetic outcome[265,266]. Locally advanced primary breast cancers, defined as Stage III, are often treated with neoadjuvant chemotherapy (discussed more in Sect 1.9) in order to shrink the primary tumor prior to surgery. Surgical management after neoadjuvant chemotherapy is similar to early stage breast cancer; however, axillary lymph node management is generally more aggressive[266,267]. Surgical management of metastatic disease (Stage IV cancer) is primarily palliative. Some studies show a survival benefit in women with metastatic breast cancer upon removal of their primary tumor, but it is difficult to generalize these data due to publication bias and the retrospective nature of the studies[268]. Surgery of metastatic lesions in the lung may be beneficial in metastatic breast cancer patients, but their main benefit is in distinguishing pulmonary metastasis from primary lung cancer in patients with a solitary lung nodule and no extra-thoracic disease[268,269]. Surgical management of hepatic nodules may be beneficial in patients with completely resectable metastatic nodules and no extrahepatic disease (except for bone metastases which are managed well with radiotherapy). However, removal of hepatic breast cancer metastases is rare, whereas hepatic colorectal carcinoma metastases are more commonly treated with surgery[268,270].

Axillary lymph node evaluation accompanies breast cancer surgery. Currently, axillary lymph node dissection is performed in all patients with clinically positive nodes, whereas patients with clinically node-negative disease undergo sentinel lymph node biopsy to evaluate the need for

axillary dissection[265]. For example, patients with stage IIIA disease may only need a sentinel lymph node biopsy, whereas patients with stage IIIB or IIIC disease often undergo initial axillary lymph node dissection, the extent of which depends on the presence of gross nodal metastases in proximal nodes[265,266]. There is no benefit to nodal dissection in patients with proven metastatic disease[266].

In summary, breast cancer surgery has evolved dramatically over the past century. Initial radical mastectomies have been replaced largely with breast-conserving surgery and subsequent breast reconstruction to achieve a cosmetic outcome. For patients with early stage breast cancer, surgery in the form of lumpectomy or mastectomy with reconstruction is recommended as the initial therapy. For patients with locally-advanced breast cancer, neoadjuvant chemotherapy is commonly used to shrink the primary tumor prior to surgery. Axillary lymph node evaluation is critical in patients with non-metastatic breast cancer and is performed at the time of surgery. For patients with clinically node-negative disease, a sentinel lymph node biopsy is used to rule out the need for axillary lymph node dissection. For patients with clinically node-positive disease, primary axillary lymph node dissection is performed to guide future treatment. Finally, surgical management of primary and metastatic tumors in metastatic breast cancer patients may provide some palliation or survival benefit in a select group of patients with favorable prognostic factors, but these patients are most commonly managed with systemic therapies.

1.8 RADIATION THERAPY

The use of radiation therapy (radiotherapy) to treat disease was implemented soon after Dr. Wilhelm Röntgen was the first to characterize X-rays in 1895[271]. Dr. Emil Grubbe is credited with

the first use of X-rays to treat recurrent breast cancer in 1896[272]. Radiation therapy has evolved dramatically over the past century and is now a critical component of breast cancer treatment.

Radiation therapy uses either particles or photons (x-rays and gamma radiation) to deliver energy to tumors in order to cause cell damage and death. Radiation can be delivered using an external beam or with an internal (brachytherapy) device. The energy transferred by particle radiation per unit distance increases with the mass of the particle used and is quantified by the linear energy transfer (LET)[273]. The higher the LET of a radiotherapy modality, the more effectively it deposits energy in the target tissue. Radiation dosages are described by total dosage amount and number of fractions. The dosage amount is described in grays (Gys), which is the number of joules per kilogram of tissue. The number of fractions describes the quantity of sessions over which the total dose of radiation is given. Recently, hypo-fractionated radiotherapy, meaning fewer fractions with more energy per fraction, has grown in favor due to fewer off-target effects on the surrounding non-tumor tissue[274].

The most common types of radiotherapy employed in modern cancer treatment are photon and charged particle beams. Photon based therapies either use X-rays (lower energy) or gamma rays (higher energy) to create secondary electrons that cause ionization and tissue damage as they travel away from the primary beam. X-ray sources include cathode ray tubes and linear accelerators, while gamma rays are generated by the decay of radioactive substances, most commonly cobalt-60, radium, and cesium[275]. Importantly, lower energy photon beams are thought to be more biologically effective against cancer due to their production of lower energy secondary electrons, which exhibit a relatively higher LET[276]. In contrast, charged particle beams use higher energy, heavier particles rather than massless photons. Examples of charged particle beams are electron, proton, neutron, and alpha-particle beams[275]. As charged particle beams are more

commonly used in radio-resistant cancers, such as prostate carcinoma and sarcomas, they will not be more extensively discussed here[273].

The main mechanism of action of radiotherapy is the ionization of molecules, which can cause tumor cell damage and death. The primary target of radiation in tumor cells are DNA molecules, which can be influenced directly or indirectly by the ionization of the DNA itself or of water molecules, which subsequently generate free radicals that induce DNA damage[277]. For X-rays and gamma rays, approximately 60% of the damage of radiation is indirect, resulting from the ionization of water and generation of free radicals[278]. DNA damage induced by radiation triggers a multitude of responses inside the irradiated cells, mediated by cytokines, growth factors, and chemokines[278]. The main therapeutic effect accomplished by these triggered responses is cell death, which can be achieved through apoptosis, mitotic catastrophe, necrosis, senescence, or autophagic cell death[275]. Moreover, induction of DNA damage in irradiated cells can release signals (cytokines, reactive oxygen species, secreted extracellular matrix proteins, etc.) that affect and kill neighboring tumor cells in a paracrine or juxtacrine manner[278]. Interestingly, while rare, primary tumor irradiation can even result in the regression of metastatic lesions, termed the abscopal effect. This effect is thought to be due to activation of anti-tumor immunity through the release of damage-associated molecular patterns (DAMPs), cytokines (such as TNF and IL-1), and chemokines (such as CXCL10) that can enhance the generation of cytotoxic T-lymphocytes[279–282].

Radiation therapy plays an extensive role in the management of both early and metastatic breast cancer and typically occurs after primary surgery and adjuvant chemotherapy. The primary use of radiotherapy is to reduce locoregional recurrence. For Stage 0, I, and II breast cancer after lumpectomy, node-negative patients receive whole breast radiotherapy (WBRT) therapy with or without a boost to the tumor bed[265]. The dosage for WBRT is 50 Gy in 23-25 fractions or 40-42.5

Gy in 15-16 fractions, for standard and hypofractionated radiotherapy respectively. Common tumor boost dosages are 10-16 Gy in 4-8 fractions[266]. For node-positive patients undergoing lumpectomy, regional radiotherapy is recommended. For patients receiving mastectomy, chest wall radiotherapy (CWRT) and irradiation of the axillary and clavicular regions are recommended in all patients, except those with a small (<5cm) primary tumor, node negative disease, and negative margin (>1mm) resections[266]. Almost all patients with stage III breast cancer will receive adjuvant WBRT (for lumpectomy) or CWRT (for mastectomy), with or without a boost to the surgical site, and supplemented by irradiation of the axillary, infraclavicular, supraclavicular, and internal mammary node regions[265,266]. Finally, radiotherapy for metastatic breast cancer is primarily palliative and is most commonly used to treat bone metastases to decrease pain and reduce the incidence of skeletal-related events[283,284].

In summary, radiation therapy has dramatically evolved over the past century and has become a staple in the treatment of breast cancer. Photon-based radiotherapy is primarily used in breast cancer, which can take the form of X-rays or gamma rays. The radiation applied by external or internal sources causes the ionization of intracellular water or DNA molecules that induce cell damage and death pathways. These effects can be both direct and indirect, the latter of which denotes the ability of irradiated cells to generate signals that cause local or distant tumor cell death. Radiotherapy is primarily used to reduce locoregional recurrence in early stage or locally advanced breast cancer, but it is also used to palliate metastatic breast lesions, most commonly those in bone.

1.9 CHEMOTHERAPY

Chemotherapy is the centerpiece in the treatment of localized and metastatic breast cancer. This chapter discusses the classes of chemotherapy relevant to breast cancer treatment, with particular emphasis placed on the mechanism of action and pharmacokinetics of each drug class. The drug classes discussed are anthracyclines (e.g. doxorubicin), alkylating agents (e.g. platins and cyclophosphamide), mitosis inhibitors (e.g. taxanes, eribulin, and vinorelbine), and antimetabolite compounds (e.g. capecitabine). Section 1.10, "Biological Agents," discusses anti-estrogen, anti-HER2, and other targeted therapies used in the treatment of breast cancer. At the end of both sections, a brief description of common systemic regimens is presented that encompasses both chemotherapies and targeted agents. A summary of chemotherapeutic agents, including their mechanisms of action and pharmacokinetic properties, is given in Table 9.

1.9.1 Anthracyclines

The first two anthracyclines were isolated from the bacterium *Streptomyces peucetius* in the early 1960s and were named doxorubicin (Dox) and daunorubicin (Dnr)[285]. Due to clinical trials in the 1980s and 1990s that demonstrated anthracycline-based chemotherapy regimens were associated with lower rates of breast cancer recurrence and increased survival, their clinical usage escalated at the end of the 20th century[286]. By 2000, approximately 70-80% of women with breast cancer under the age of 70 were receiving anthracycline-based chemotherapy regimens[286]. In order to improve efficacy and reduce toxicity, other anthracyclines were synthesized, with epirubicin (Epi) and idarubicin (Ida) garnering FDA approval. Other anthracyclines, such as pirarubicin and mitoxantrone, have been studied in clinical trials but have demonstrated more severe cardiac

toxicity than desirable[285]. Currently, Dox and Epi are FDA-approved and clinically used for the treatment of early and metastatic breast cancer, whereas Dnr and Ida are both used in the treatment of leukemias[287], so will not be further discussed.

Table 9. A summary of chemotherapeutic agents used in the treatment of breast cancer

Compound	Mechanism(s) of Action	Mechanisms of Resistance	$T_{1/2}$ (h)	Toxicities
Doxorubicin **Epirubicin**	TopII inhibition DNA damage	↓ Erk, Cyclin D ↑ Cols 1, 12, 21, TGFβ1	34 30	Cardiac Muco-cutaneous Myelosupp
Cyclophosphamide	DNA crosslinks	↑ALDH ↑GST	2-8	Hemorrhagic myositis and cystitis BLC and Leukemias
Cisplatin **Carboplatin**	DNA crosslinks	↑ NER ↑E-cad, ZEB1, Akt	0.5 2	Neurotoxicity Myelosupp
sb-Paclitaxel **nab-Paclitaxel** **Docetaxel**	Microtubule stabilization	↑ MDR1, MRP2,7 ↑ β3-tubulin	20.5 21.6 13.5	Neutropenia Neurotoxicity Febrile neutropenia
Ixabepilone	Microtubule stabilization	β1-tubulin polymorphisms	52	Neutropenia Neurotoxicity
Eribulin	Microtubule destabilization	↑ MDR1, MRP8	40	Neutropenia Neurotoxicity
Vinorelbine	Microtubule destabilization	↑ MDR1, MRP7	35	Neutropenia
5-FU **Capecitabine**	TS inhibition DNA/RNA Inc.	↑ TS, DPD	0.25 0.75	Myelosupp, GI, HFS Like 5-FU, but milder
Gemcitabine	DNA Inc. RNR inhibition	↑ PI3K/Akt, NF-κB	0.25	Neutropenia Thrombocytopenia

$T_{1/2}$ = elimination half-life; Myelosupp = Myelosuppression; ALDH = aldehyde dehydrogenase; Col = Collagen; TopII = Topoisomerase II; GST = glutathione S-transferase; BLC = bladder carcinoma; NER = nucleotide excision repair; E-cad = E-cadherin; sb = solvent bound; nab = nanoparticle albumin bound; 5-FU = 5-Fluorouracil; Inc. = incorporation; TS = thymidylate synthase; DPD = dihydropyrimidine dehydrogenase; GI = gastrointestinal; HFS = hand-foot syndrome; RNR = ribonucleotide reductase.

Anthracyclines work by several mechanisms of action. Since Dox and Epi demonstrate equivalent efficacy against breast cancer, Dox will be used to illustrate the mechanisms by which

anthracyclines exert anti-tumor effects. Importantly, Dox efficacy is dependent on nuclear localization, which is guided by the 26S proteasome. Dox binds to the 20S subunit of the proteasome with high affinity, which is then translocated into the nucleus through the nuclear pores[288]. Once in the nucleus, Dox avidly binds to the DNA due to its higher affinity for DNA than the 20S subunit of the proteasome[288]. This mechanism can explain the preferential accumulation of doxorubicin in proliferating cells, as these cells often demonstrated enhanced nuclear localization of the proteasome[288]. Moreover, Dox binding to the 20S subunit of the proteasome reduces its protease activity and promotes the accumulation of ubiquitinated proteins, which can induce apoptosis in tumor cells to a similar degree as proteasome inhibitors[289]. Resistance to Dox in breast cancer is mediated by the alteration of proliferative gene expression (e.g. Cyclin D and Erk) and extracellular matrix molecules, such as collagens 1, 12, 21, and TGFβ1[290–292].

Once in the nucleus, the main mechanism of action of Dox is inhibition of the nuclear enzyme topoisomerase II (TopII)[285] . Physiologically, TopII acts to untangle DNA helices by binding to the DNA, inducing controlled double stranded breaks, and subsequently ligating these breaks after the unknotting is completed[293]. Dox stabilizes the TopII – DNA complex to inhibit the ligation of formed DNA breaks, which subsequently results in enhanced DNA cleavage by the enzyme[294]. The DNA damage that accumulates results in growth arrest in either G_1 or G_2, with subsequent activation of intracellular apoptosis pathways[295–297]. Moreover, Dox resistance has been observed in cells with altered expression of TopII, and the genetic status of the TopIIα gene is predictive of the clinical response to anthracycline based therapy[285,298,299].

The second mechanism by which anthracyclines act involves DNA damage through TopII-independent mechanisms. The central ring of Dox and other anthracyclines is a quinone moiety that can generate free radicals upon reduction to a semiquinone[300]. These free radicals can induce

DNA damage either directly, through lipid peroxidation and subsequent formation of DNA adducts, or through the formation of oxidative base lesions[285]. Moreover, free radical reactions can modify Dox and allow it to directly intercalate into DNA[301]. Finally, Dox treatment has been shown to induce p53 and reduce telomerase activity, which can induce cell death or senescence[302].

The pharmacokinetics of Dox and Epi are similar. Both medications are dosed intravenously (IV), meaning the bioavailability is 100%. The tissue distribution of Dox and Epi is rapid, with a plasma elimination half-life under 5 minutes[303]. This initial tissue uptake is succeeded by two longer tissue elimination phases, with a terminal half-life of approximately 34 and 30 hours for Dox and Epi respectively[303,304]. Clearance of Dox, Epi, and their metabolites is primarily through biliary excretion, with some urinary excretion that is observed more prominently with Epi. Hepatic metabolism of Dox and Epi is predominantly through CYP3A4 and CYP2D6[305]. The main toxicities of Dox and Epi are myelosuppression, mucosal and cutaneous toxicities, and cardiotoxicity. Cardiotoxicity typically presents as dilated cardiomyopathy in adults and is less common with Epi than Dox[306]. Liposomal-encapsulation of Dox has been shown clinically to increase the half-life and decrease the toxicity of the parent drug[307,308].

1.9.2 Alkylating Agents

The two classes of alkylating agents used in the treatment of breast cancer are nitrogen mustards and platinum-based chemotherapeutics (“platins”). Cyclophosphamide is the only clinically approved nitrogen mustard for breast cancer, whereas two platins, cisplatin and carboplatin, are approved. Cyclophosphamide and platins will be discussed in more detail below, specifically focusing on their mechanism of action and pharmacokinetics.

1.9.2.1 Cyclophosphamide

The first use of chemotherapeutic agents was in 1942 when a Polish immigrant was treated with nitrogen mustard for lymphosarcoma[309]. The only clinically used alkylating agent in breast cancer is cyclophosphamide (Cpa). Cpa was first clinically implemented in 1958 and FDA approved one year later[310,311]. All alkylating agents act by the addition of an alkyl group to DNA bases, most preferentially guanine. These added groups form intra- and inter-strand linkages that inhibit the ability of DNA polymerase to replicate the DNA, resulting in growth arrest and apoptosis[312]. Moreover, these alkylation events can also form DNA-DNA and DNA-protein crosslinks, with the former being shown to be the mechanism of cellular toxicity[310]. These adducts lead to induction of apoptotic pathways, ending with caspase and PARP cleavage and cell death[313,314].

Resistance to Cpa is multifactorial. First, aldehyde dehydrogenase (ALDH) plays a significant role in resistance to Cpa[315,316]. Increased ALDH activity is associated with Cpa resistance and can be reversed with the ALDH inhibitor disulfiram[315]. Second, altered expression of genes involved with apoptosis, such as p53 and caspase-3, can induce resistance to Cpa[316,317]. Third, increased synthesis of glutathione, by alterations in glutathione S-transferases, can reduce the efficacy of Cpa by quenching its reactivity towards DNA[317–319].

The pharmacokinetics of Cpa begins with oral or IV administration, followed by a necessary activation step in the liver, predominantly accomplished by CYP2B6 and CYP3A4[312,319]. Oral and IV administration achieve near equivalent serum concentrations, as Cpa has 85-100% oral bioavailability[310]. Cpa is distributed into total body water with minimal fat distribution[320], which results in an elimination half-life of 2-8 hours depending on dosage[310]. Cpa is primarily excreted in the urine as metabolites of the activated drug[321]. Inactivation of Cpa is primarily accomplished by cellular ALDH, most predominantly by the isoform ALDH1A1[311]. The

dose-limiting toxicity for Cpa is hemorrhagic myocarditis, with some transient minor cardiac toxicity observable in up to half of patients[311]. Other significant side effects are hemorrhagic cystitis, development of secondary malignancies such as bladder cell carcinoma and leukemias, and gonadal failure[311].

1.9.2.2 Platinum-containing drugs

Platinum-based chemotherapeutics ("platins") act as alkylating-like compounds. Platins covalently bond to DNA and generate intra- and inter-strand crosslinks, which interfere with DNA repair, causing growth arrest and apoptosis[322]. There are two platins used in the treatment of breast cancer: cisplatin (Cis) and carboplatin (Crb). Cis and Crb behave very similarly, forming identical DNA adducts, but with the latter exhibiting lower potency and toxicities[323,324]. The most common cause of cytotoxicity with platins is the formation of intra-strand G-G adducts, constituting about 90% of all adducts. Importantly, cells with dysfunctional DNA repair are more susceptible to platin therapy[325]. Cisplatin has multiple other mechanisms of action, such as the formation of reactive oxygen species and lipid peroxidation. These factors and genomic instability cause the activation of the intrinsic apoptotic pathway, in part mediated by DNA damage response proteins such as p53, and result in cell death[322].

Resistance to Cis occurs through a multitude of mechanisms, with altered expression of genes that mediate apoptosis, DNA damage-repair, the cell cycle, cytoskeletal proteins, small GTPases, and transcription factors[326–328]. In particular, the nucleotide excision repair (NER) pathway is primarily responsible for repairing adducts caused by Cis[329]. Additionally, alterations in membrane transporters, such as copper transporter 1 (CTR1) and efflux ATPases ATP7A and ATP7B, play a significant role in Cis resistance[326,329]. Moreover, E-cadherin expression and related survival pathways, such as Akt, have been shown to increase resistance to Cis[330,331]. Finally,

increased expression of zinc finger E-box binding homeobox 1 (ZEB1) has been shown to increase Cis resistance by activating ATM-initiated homologous recombination-mediated DNA damage repair[332].

Both Cis and Crb are administered intravenously. After administration, Cis and Crb are activated by hydrolysis of chloride or cyclobutane groups respectively[323]. Cis and Crb have similar volumes of distribution and excretion profiles, with both compounds demonstrating predominantly renal clearance, with elimination half-lives of 0.5 and 2 hours respectively[333–335]. In contrast to Crb, Cis can be metabolized in the proximal tubule of the kidney into a renal toxic metabolite[336]. Generally, Crb has lower toxicity than Cis due to its lower reactivity. Common toxicities with platins include nephrotoxicity, neurotoxicity, myelosuppression, hepatotoxicity, and cardiotoxicity[322,323]. In particular, the most severe dose-limiting toxicities for Cis and Crb are neurotoxicity and myelosuppression respectively. Cis causes permanent hearing loss in 40-80% of treated patients and has been detected in the cochlea years after treatment[337]. Approximately 85% of patients with a cumulative Cis dose of greater than 300 mg/m^2 have some degree of peripheral sensory neuropathy[323]. Crb causes severe thrombocytopenia and neutropenia in 25% and 18% of patients respectively, which are fortunately reversible upon cessation of treatment[323].

1.9.3 Mitotic Inhibitors

The dynamic assembly and disassembly of microtubules is essential for mitosis. All mitotic inhibitors used in the treatment of breast cancer act to inhibit the role microtubules play in cell division. These drugs include the taxanes, eribulin, ixabepilone, and vinorelbine. Inhibition of microtubule action can occur either by stabilizing their polymerization or by promoting their turnover[338]. Taxanes and ixabepilone bind to the Taxol-domain of β-tubulin and function as

microtubule-stabilizing agents, whereas eribulin and vinorelbine bind to the vinca-domain of β-tubulin and function as microtubule-destabilizing agents[339]. While the taxane drug class is used for management of both primary and recurrent/metastatic breast cancer, eribulin, ixabepilone, and vinorelbine are solely used for the latter[339]. For this reason, the taxane drug class will be discussed more extensively, as it is used in the treatment of all stages of breast cancer. Subsequently, eribulin, ixabepilone, and vinorelbine will be covered together in Section 1.9.3.2, "Other Microtubule Inhibitors."

1.9.3.1 Taxanes

The incorporation of taxanes in the management of breast cancer in the mid-1990's dramatically improved the outcomes of patients with both early and late stage breast cancer[340,341]. The two clinically used taxanes in the management of breast cancer are paclitaxel (Ptx) and docetaxel (Dtx), with higher efficacy and toxicity seen in the latter due to higher affinity for β-tubulin and a longer intracellular retention time[338,341]. Both Ptx and Dtx act to induce cell cycle arrest and apoptosis by binding to β-tubulin, which results in microtubule polymerization, existing tubule stabilization, and a G_2/M phase arrest[338,339,342,343]. Since Dtx exhibits a higher affinity for β-tubulin, it can also inhibit centrosome organization and exhibit cytotoxic effects on S-phase cells[338]. Finally, Ptx and Dtx can directly induce apoptosis through phosphorylation of Bcl-2, which is achieved at a 100-fold lower concentration with Dtx than Ptx[344].

There are four main mechanisms of resistance to taxanes that have been uncovered *in vitro*, though the clinical significance of these mechanisms as biomarkers to guide therapy is still unclear[345]. The primary mechanism of resistance involves the expression of membrane efflux pumps, such as MDR1 (P-glycoprotein), MRP2, and MRP7[339,346]. A secondary mechanism of resistance to taxanes is an alteration of the expression of or mutations in the β-tubulin isotype

genes. Most predominantly, increased expression of β3-, βV-, and βII-tubulin are associated with taxane resistance[347]. Furthermore, mutations in tubulin genes have been detected in cell lines resistant to taxanes, but the low clinical prevalence of these mutations precludes their use as biomarkers[348]. Third, some studies suggest taxane resistance is associated with mutations in or altered expression of apoptotic signaling proteins, such as P53, Bcl-2, Bcl-xL, and NF-κB[339], yet there also exist conflicting reports[345]. Finally, both *in vitro* and clinical taxane resistance is associated with a deficiency in spindle assembly checkpoint proteins, such as Mad2 and BubR1, which suggests checkpoint functionality is required for taxane sensitivity[349,350].

Ptx and Dtx have substantial differences in pharmacokinetics. Both compounds are administered by IV infusion. There are two formulations of Ptx: solvent-based (most commonly Cremophor EL) and nanoparticle albumin bound (nab). Both formulations exhibit non-linear pharmacokinetics, meaning dosage changes result in a disproportionate change in the total drug exposure[338,351]. Solvent-based Ptx (sb-Ptx) is infused over 3 hours, whereas nab-Ptx is administered over 30 minutes[352]. The main pharmacokinetic difference between sb-Ptx and nab-Ptx is that unbound Ptx concentration and cellular transport is higher in the latter, which results in a higher tumor response rate and overall survival[351,353]. Additionally, nab-Ptx is void of Cremophor EL – mediated toxicities, such as hypersensitivity and neurotoxicity[354]. In contrast, Dtx is administered over one hour and exhibits linear (proportional) pharmacokinetics[338]. Both Ptx and Dtx are extensively metabolized by CYP450 enzymes, most predominantly by CYP3A4, and biliary excretion is responsible for >95% of drug clearance[338,355,356]. The elimination half-life is slightly lower for Dtx, with values of 20.5, 21.6, and 13.5 hours for sb-Ptx, nab-Ptx, and Dtx respectively[357,358]. The dose-limiting toxicities for sb-Ptx and nab-Ptx are neuropathy and neutropenia, with sb-Ptx demonstrating a higher incidence of neutropenia but a lower incidence of

neuropathy compared to nab-Ptx[359]. In contrast, the main dose-limiting toxicities for Dtx is febrile neutropenia[360]. Other side effects seen with Dtx are fluid retention, nail toxicity, pneumonitis, and neuropathy, with the incidence of the last much lower than observed with Ptx[360].

1.9.3.2 Other Microtubule Inhibitors

While anthracyclines and taxanes have greatly improved the overall survival of women with breast cancer, recurrent metastatic breast cancer remains difficult to treat. In particular, due to the increasing use of anthracyclines and taxanes in the adjuvant setting, ixabepilone, eribulin, and vinorelbine are playing more significant roles in the management of metastatic breast cancer. The mechanism of action, pharmacokinetics, and toxicity of these drugs will be described in the next few paragraphs.

Ixabepilone acts similarly to paclitaxel by stabilizing microtubules through binding β-tubulin at the Taxol domain. Currently, ixabepilone is approved as mono- and combination therapy with capecitabine for the treatment of taxane and anthracycline resistant, recurrent breast cancer[361]. In contrast to paclitaxel, ixabepilone can bind to multiple isomers of β-tubulin, including the isomer β3 to which paclitaxel binds[316]. Due to the multiple sites at which ixabepilone can bind, it has been shown to be effective in taxane-resistant metastatic breast cancer. Additionally, ixabepilone exhibits low activity as a substrate for drug transporters often overexpressed in taxane resistance, such as MDR1[362]. Mechanisms of resistance to ixabepilone are still poorly characterized, but one proposed mechanism involves polymorphisms in β1-tubulin[363]. Ixabepilone is excreted primarily through the bile (65%), both unchanged and as metabolites formed through CYP3A4[364], and urine (21%) with an elimination half-life of 52 hours[365]. The most common dose-limiting toxicities observed are neutropenia and neuropathy, with a lower incidence of neuropathy as compared to taxanes[366].

Eribulin acts as a microtubule destabilizing agent through binding β-tubulin at the Vinca domain, limiting microtubule growth, and causing mitotic catastrophe, resulting in cell death[367]. Eribulin is currently approved as monotherapy for extensively pretreated metastatic breast cancer[368,369]. In contrast to paclitaxel, eribulin efficacy is inversely proportional to β3-tubulin abundance[370]. In addition to destabilizing microtubules, studies have shown that eribulin can induce tumor vascular remodeling and reversal of EMT, which may play roles in its overall survival benefit observed in metastatic breast cancer[367]. Resistance to eribulin is often associated with upregulation of MDR1 or MRP8[371,372]. While eribulin is a substrate of CYP3A4 *in vitro*, the majority of the drug is excreted unchanged in the bile (61%), a minority unchanged in the urine (8%), and <1% of drug in the plasma is in the form of metabolites[373]. The elimination half-life is 40 hours[373]. The most common dose-limiting toxicities observed are neutropenia and neuropathy. While high grade neutropenia is observed in almost half of patients, febrile neutropenia is only seen in 3% of patients, much lower than that observed with docetaxel treatment (up to 40%)[367,374].

Vinorelbine acts as a microtubule destabilizing agent through binding β-tubulin at the Vinca domain, inhibiting the formation of the mitotic spindle, which results in mitotic catastrophe and cell death[375]. Vinorelbine shows high specificity for mitotic microtubules, which precludes the neurotoxicity commonly seen with microtubule agents[376]. The use of vinorelbine in metastatic breast cancer is becoming more common as a second line therapy after anthracycline and taxane treatment[366,377]. Beneficially, vinorelbine has both IV and oral formulations, the latter of which is useful for patients desiring fewer hospital visits[377]. Mechanisms of resistance to vinorelbine include upregulation of MDR1 and MRP7[378,379]. Vinorelbine extensively binds to platelets in the plasma and is metabolized by CYP3A4[380,381]. The majority of vinorelbine is excreted in the bile (30-60%), a minority in the urine (20%), and metabolites are present in very low concentrations in

the plasma[381]. The elimination half-life is approximately 35 hours[381]. The most common dose-limiting toxicity observed is neutropenia, observed in 40% of patients, with <5% associated with a febrile event[377].

1.9.4 Antimetabolite Drugs

The three antimetabolite drugs used in the treatment of breast cancer are 5-fluorouracil (5-FU), capecitabine (Cap), and gemcitabine (Gem). Whereas 5-FU is solely used in combination chemo regimens for both early and late stage breast cancer, Cap and Gem are both approved as single agents or in combination with targeted agents for metastatic breast cancer[265].

1.9.4.1 5-Fluorouracil and Capecitabine

The drugs 5-FU and Cap both belong to the fluoropyrimidine drug class. The use of 5-FU and Cap differ, in that the former is FDA-approved for both early and metastatic breast cancer, whereas the latter is only approved for anthracycline and/or docetaxel resistant metastatic breast cancer[265,382]. Cap is an oral prodrug of the IV administered 5-FU. The enzymatic conversion of Cap to 5-FU involves the following three steps: 1) cleavage by carboxylesterase in the liver, 2) deamination by cytidine deaminase, and 3) processing by thymidine phosphorylase to generate 5-FU[383]. Importantly, the enzymes important for steps 2 and 3 of this conversion have the highest activity in the liver and tumor tissue, which makes Cap most selectively activated in these tissues[384]. Once 5-FU is generated, it undergoes either anabolic (activating) or catabolic (deactivating) metabolism, which dictate its mechanism of action.

Both 5-FU and Cap have several mechanisms of action. First, three phosphorylation steps of 5-FU generate 5-FUTP (5-fluorouracil triphosphate), which is recognized as UTP (uracil

triphosphate) and is capable of incorporating into all classes of RNA and exerting damaging effects on RNA processing, cellular metabolism, and cell viability[385]. Second, 5-FU can be converted into 5-FdUMP (5-fluorodeoxyuridine monophosphate), which is a potent competitive inhibitor of thymidylate synthase (TS), critical to the intracellular synthesis of thymidine[383]. Thus, abundance of 5-FdUMP reduces the formation of dTMP (deoxythymidine monophosphate) and reduces the availability of this critical nucleotide for DNA replication and repair, which can facilitate activation of apoptotic pathways[383]. Finally, phosphorylation of 5-FdUMP into 5-FdUTP allows for direct incorporation of 5-FU into DNA, which induces DNA damage, cell cycle arrest, and attempted repair through the base excision repair pathway[386].

Inactivation of 5-FU is accomplished by a three step catabolic pathway, the first and rate limiting step catalyzed by the enzyme dihydropyrimidine dehydrogenase (DPD). Further enzymatic steps generate further metabolites, which eventually produce FBAL (α-fluoro-β-alanine). Mechanisms of resistance to 5-FU and Cap include increased expression of DPD and TS[383,387–389].

While 5-FU is given as either an IV bolus or continuous infusion over a 24 hour period, Cap is an oral drug with extensive absorption in the GI tract, followed by subsequent modification in the liver[383,390]. As discussed above, metabolism of Cap into 5-FU and subsequent anabolic steps result in its activation. Both 5-FU and its metabolites undergo almost entirely urinary excretion, with an elimination half-lives of 15 minutes and 45 minutes for 5-FU and Cap respectively[390,391]. The main toxicities of 5-FU depend on the method of administration. For bolus IV injection of 5-FU, the most common toxicities are myelosuppression, oral mucositis, and gastrointestinal (GI) toxicity (diarrhea), the latter mostly due to the phosphorylation of 5-FU in the gut epithelium[383,392]. In contrast, for continuous infusion of 5-FU, toxicities include hand-foot syndrome and a lower

incidence of myelo- and GI-toxicity[383]. Infrequent, reversible cardiotoxicity and neurotoxicity can also be observed with 5-FU treatment. The preferential accumulation and activation of Cap in tumor tissue results in a more mild toxicity profile than is observed with 5-FU[390,392]. Importantly, Cap is an inhibitor of CYP2C9, so drug interactions with warfarin and phenytoin have been reported[393].

1.9.4.2 Gemcitabine

Gemcitabine (Gem) exerts its cytotoxic effects through insertion into the DNA as an analog of cytosine. Similarly to 5-FU, Gem requires intracellular phosphorylation by deoxycytidine kinase for activity[394]. There are two main cytotoxic mechanisms of action of Gem that interact with each other. First, gemcitabine triphosphate competes with deoxycytidine triphosphate (dCTP) for incorporation into DNA, which inhibits DNA synthesis and initiates DNA repair and apoptosis pathways[395]. Second, gemcitabine diphosphate is a potent inhibitor of ribonucleotide reductase, which prevents the synthesis of dCTP, thus potentiating the ability of gemcitabine triphosphate to be incorporated into DNA[395,396]. Breast cancer resistance to Gem is seen with upregulation of survival signaling pathways, such as PI3K/Akt and NF-κB, though typically is less common than other chemotherapeutic agents, such as anthracyclines and taxanes[397,398].

Gemcitabine is given as a short (30 minute) infusion for the treatment of metastatic breast cancer. Due to rapid deamination, Gem has a short elimination half-life of only 15 minutes. Its deaminated byproduct, difluoro-deoxyuridine (dFdU), has a much longer half-life of 50 hours[399]. Gem and its metabolites primarily undergo renal excretion[399]. Gem typically has a lower toxicity profile than other agents used in the management of metastatic breast cancer, with the most common dose-limiting toxicities being neutropenia and thrombocytopenia[396].

1.10 BIOLOGICAL AGENTS

Targeted therapies play a large role in the treatment of breast cancer. Approximately 70-80% of breast cancers are estrogen receptor positive, and 15-20% of breast cancers are HER2 type [76,80,81]. As such, anti-estrogen and anti-HER2 based therapies are commonly used in conjunction with the chemotherapeutic agents discussed in the previous sections. Similarly, a select group of cancers are responsive to therapies that target EGFR, cyclin-dependent kinases 4 and 6 (CDK4 and CDK6), poly-ADP-ribose polymerase (PARP), and the checkpoint proteins programmed death-1 and -ligand 1 (PD-1/PD-L1). The mechanism of action and pharmacokinetics of each of these drug classes are discussed below in Table 10.

Table 10. A summary of biological agents used in the treatment of breast cancer

Compound	Drug Class	Mechanisms of Resistance	$T_{1/2}$ (h)	Toxicities
Tamoxifen	SERM	↑ EGFR/HER2, SCR3, ERβ	168	EC, TED, cataracts
Raloxifene		↓ CYP2D6/3A4, ERα (or mut)	27.7	TED
Fulvestrant	SERD	↑ PI3K/MAPK, FOXA1, NF-κB ↓ ERα (or mut)	1176	Nausea, asthenia, pain
Letrozole	Aromatase Inh	↑ EGFR/HER2, ERα Sens.	42	Osteoporosis, fracture, ischemic CVEs
Anastrazole		p53 mut	41	
Exemestane		↓ ERα (or mut)	27	
Trastuzumab	HER2 Inh	HER2 mut, ↑ PI3K/Akt, EGFR/HER3, FcγR mut	672	↓ LVEF, pneumonitis
T-DM1	HER2 Inh, MTI	↓ HER2, HER2 shedding	96	Thrombocytopenia ↓ LVEF
Pertuzumab	HER2 Inh	HER2 shedding, ↑ PI3K/Akt, EGFR/HER3	456	
Lapatinib	HER2/EGFR Inh	HER2 mut, ↑ HER3, Akt	24	Diarrhea, rash
Palbociclib	CDK4/6 Inhibitor	↑ Cyclin E, $p16^{Ink4a}$ ↓ Rb	26	Neutropenia
Ribociclib			36	Neutropenia
Abemaciclib			17-38	Fatigue

$T_{1/2}$ = elimination half-life; SERM = selective estrogen receptor modulator; EC = endometrial carcinoma; TED = thromboembolic disease; mut = mutation; SERD = selective estrogen receptor degrader; Sens. = sensitivity LVEF = left ventricular ejection fraction; Inh = Inhibitor; MTI = microtubule inhibitor; FcγR = Fc-gamma receptor; CHF = congestive heart failure; CDK4/6 = cyclin dependent kinase 4 and 6.

1.10.1 Anti-Estrogen Therapies

Most breast cancers are ER positive, so anti-estrogen therapies play a significant role in medical management. There are four main classes of anti-estrogen therapies used in the management of estrogen receptor positive breast cancer: selective estrogen receptor modulators (SERMs), selective estrogen receptor degraders (SERDs), aromatase inhibitors (AIs), and luteinizing hormone releasing hormone (LHRH) analogues.

1.10.1.1 Selective Estrogen Receptor Modulators (SERMs)

Selective estrogen receptor modulators (SERMs) have been clinically approved for the treatment of breast cancer for four decades. Initially discovered as a non-steroidal anti-estrogen compound and used unsuccessfully as a contraceptive, tamoxifen was first clinically tested in metastatic breast cancer in 1971 and subsequently approved in 1977[400]. The success with tamoxifen motivated the synthesis of similar drugs, with the discovery of raloxifene in 1982[401]. Subsequently, studies in the 1980s, after advances in cloning of the ERα gene, suggested tamoxifen and raloxifene worked by selectively modifying ER signaling[400]. Investigation of tamoxifen for the chemoprevention of breast cancer began in 1985, with eventual approval of tamoxifen for breast cancer risk reduction in 1998[400,402]. Raloxifene was approved for the treatment and prevention of osteoporosis in postmenopausal women in 1998[403]. Subsequently, clinical trials demonstrated equivalence between raloxifene and tamoxifen in breast cancer risk reduction, granting raloxifene a second approval for the chemoprevention of breast cancer in 2007[404–406]. Importantly, raloxifene is not approved for the treatment of breast cancer, only for risk reduction in postmenopausal women[406,407].

The three most pertinent sites of action for SERMs are those in which estrogen signaling plays a significant role in organ pathophysiology, namely in the breast, the bone, and the uterus. Importantly, tamoxifen and raloxifene exhibit different tissue sites of action. While tamoxifen and raloxifene act as anti-estrogens in breast carcinoma and as pro-estrogens in the bone, only tamoxifen acts as a pro-estrogen in the uterus[408,409]. Thus, while both tamoxifen and raloxifene share similar effects on breast cancer and osteoporosis prevention, only tamoxifen increases the clinical risk for endometrial carcinoma[407–409].

The tissue dependence of SERM activity is intimately involved with their ability to influence estrogen receptor conformation and binding to coregulatory molecules. While the SERMs bind in the same pocket as estradiol, the binding of tamoxifen, raloxifene, and estradiol induce different conformations of the estrogen receptor[409–411]. Additionally, the interaction of coactivators and corepressors with ligand-bound estrogen receptor is dependent on the ligand. Coregulator interactions with the estrogen receptor are mediated through the AF1 and AF2 domains, which are regulated by MAPK activity and estradiol binding, respectively[412]. Importantly, SERMs primarily alter signaling through the AF2 domain and exhibit variable effects on the AF1 domain responsible for estrogen agonistic activity[413]. For example, SRC-1 is a coactivator that mediates estrogen agonistic effects of both estradiol and tamoxifen but is required for agonist activity of the latter[414]. Similarly, the corepressor SMRT can reduce the agonist activity of tamoxifen but not estradiol[414]. Moreover, the relative abundance of the estrogen receptor isoforms ERα and ERβ can guide the activity of the SERMs in affecting gene transcription[415]. Finally, the agonist/antagonist effects of SERMs can be influenced by intracellular signaling networks, such as protein kinase A[416]. In summary, the mechanism of action of SERMs depends

on the relative abundance of the target receptor isoforms, presence of coregulators, and activation of intracellular signaling networks.

Mechanisms of resistance to SERMs can be classified as either intrinsic or acquired. Intrinsic resistance to SERMs is most often due to crosstalk from other signaling pathways or differences in drug metabolism. For example, HER2 overexpression results in increased estrogen-independent ER signaling, making tumors resistant to *de novo* SERM treatment[417–419]. Intrinsic resistance to tamoxifen can also be observed in patients with polymorphisms in CYP2D6 and CYP3A4, the two CYP450 isozymes responsible for metabolic activation of tamoxifen[418,419]. Acquired resistance to tamoxifen often involves alterations in the expression of estrogen receptor isoforms, coregulators, and activity of other intracellular signaling networks. Increased expression of ERβ and loss of ERα have been observed in tamoxifen-resistant breast cancer[420,421]. Similarly, mutations in ERα are common drivers of resistance[420,422]. Moreover, expression of SRC3, an ERα co-activator expressed in over 50% of breast tumors, can increase the estrogen agonist effects of tamoxifen, which leads to resistance[412]. Finally, overexpression of HER2 or EGFR act as a mechanisms of acquired resistance to tamoxifen by enhancing Erk1/2 and Akt activity[420,423].

Tamoxifen is administered orally and produces peak serum levels after 3-6 hours and steady state levels after 4 weeks[424]. Tamoxifen is extensively bound to albumin (~98%), which results in a long elimination half-life of 7 days[425]. Tamoxifen is extensively metabolized by the CYP450 system, which is required for its activation. The two primary active metabolites of tamoxifen are endoxifen and 4-hydroxytamoxifen, with the former found at more than 10-fold higher concentrations in patient serum[426]. The two main CYP450 isoforms that are responsible for tamoxifen metabolism are CYP2D6 and CYP3A4[424]. Tamoxifen is eliminated primarily by biliary excretion, with polar conjugates of tamoxifen and its metabolites formed through glucuronidation,

accounting for 75% of the excreted drug[426]. The most common toxicities with tamoxifen are a 2-5 fold increase in uterine cancer, a 2-3 fold increase in thromboembolic disease (only seen in postmenopausal women), hot flashes, and vaginal discharge[407,427]. Additionally, patients receiving tamoxifen are at a slightly greater risk for the formation of cataracts[407,427].

Raloxifene undergoes significant first pass metabolism by glucuronidation, resulting in only 2% of the drug dose reaching the plasma[428]. Similar to tamoxifen, raloxifene is extensively plasma protein bound (95%), yet has a much lower elimination half-life at 27.7 hours[428]. The metabolism of raloxifene is primarily accomplished by glucuronidation without involvement of the CYP450 ensemble[428]. Similar to tamoxifen, raloxifene primarily undergoes biliary excretion. Raloxifene has fewer adverse effects than tamoxifen, yet still results in a 3 fold increase in risk for thromboembolic disease[409].

1.10.1.2 Selective Estrogen Receptor Degraders (SERDs)

Selective estrogen receptor degraders (SERDs) were pioneered to overcome endocrine-resistant breast cancer through targeting ERα, which commonly plays a role in tamoxifen-resistant disease[429]. Currently, fulvestrant is the only SERD approved for the treatment of locally advanced and metastatic ER+ breast cancer, either as monotherapy for endocrine therapy naive disease or as monotherapy or combination therapy for endocrine-resistant breast cancer[430]. The success of fulvestrant and its inconvenient delivery method (intramuscular injection) have motivated the development of other orally bioavailable SERDs, which have shown promising preclinical efficacy and are currently being investigated in clinical trials[429,431].

Fulvestrant is an estradiol analogue that interferes with estrogen receptor signaling through multiple mechanisms of action. Fulvestrant is a competitive antagonist of the estrogen receptor, with a binding affinity similar to that of estradiol[432]. Additionally, fulvestrant-bound estrogen

receptor shows impaired dimerization and nuclear localization[433]. Moreover, fulvestrant binding induces conformational changes in the estrogen receptor that disable both AF1 and AF2, in contrast to tamoxifen, which primarily disables AF2 binding[432]. This means that fulvestrant demonstrates no estrogen agonist activity[434,435]. Finally, fulvestrant increases turnover of the estrogen receptor by targeting it to the proteasome for degradation[436–438]. Mechanisms of resistance to fulvestrant include estrogen receptor downregulation or mutation, activation of PI3K and MAPK, and activation of NF-κB and FOXA1[438–440].

Fulvestrant is most commonly given as a single intramuscular (IM) injection of 500mg. In comparison to IV dosing, IM administration results in a long-acting delivery of fulvestrant, with an elimination half-life of approximately 49 days[441]. Fulvestrant is primarily metabolized by sulfate conjugation, and while it is a substrate for CYP3A4 metabolism, this does not play a major role in drug clearance[442]. Fulvestrant is generally well tolerated, with the most common adverse events being nausea, asthenia, pain, and headache[435].

1.10.1.3 Aromatase Inhibitors

Aromatase inhibitors have several uses in the management of postmenopausal women with estrogen receptor positive breast cancer. First, in postmenopausal women with a high risk of developing breast cancer, aromatase inhibitors are effective at reducing the incidence of, but not the mortality of, breast cancer[443]. Second, neoadjuvant use of aromatase inhibitors has been shown to be as effective as tamoxifen and in some groups of patients more effective, in particular in patients with triple positive ($ER^+/PR^+/HER2^+$) disease[444]. Third, adjuvant use of aromatase inhibitors is recommended in place of tamoxifen, due to clinical trials that demonstrated lower recurrence with adjuvant aromatase inhibitors versus tamoxifen[445]. Finally, aromatase inhibitors

are used in the context of metastatic breast cancer, often as combination therapy with other estrogen targeted therapies, such as fulvestrant[446].

Aromatase inhibitors reduce circulating estrogens by preventing the peripheral conversion of androgens to estrone. In premenopausal women, granulosa cells in the ovaries synthesize estradiol from cholesterol, which circulates as the predominant form of natural estrogens[447]. In postmenopausal women, the ovaries have lost the ability to synthesize estradiol, necessitating the peripheral conversion of androgens into estrone, which serves as the primary source of natural estrogens. This peripheral conversion is facilitated by the enzyme aromatase, a member of the CYP450 family and the product of the CYP19 gene found in adipose, skin, bone, breast, and brain tissue[447,448]. Since postmenopausal women cannot synthesize estradiol, newer selective aromatase inhibitors are able to reduce circulating estrogens by over 95%[448–450]. There are three clinically used aromatase inhibitors - letrozole, anastrazole, and exemestane – which all act by blocking aromatase, although they vary slightly in chemical structure.

Exemestane is a steroidal aromatase inhibitor whose structure is derived from androstenedione, the natural substrate of aromatase, which binds irreversibly to the enzyme[451]. In contrast, letrozole and anastrazole are non-steroidal aromatase inhibitors, which are derived from phenobarbitone and bind reversibly to the enzyme[451]. While all three aromatase inhibitors achieve similar decreases in plasma estrogen levels and increases in bone resorption, exemestane also induces bone formation due to its androgen likeness[452]. Some data suggests that anastrazole may be more selective for aromatase than letrozole[453].

Resistance to aromatase inhibitors can occur by multiple pathways. First, the estrogen receptor can develop hypersensitivity to estrogen, allowing even the miniscule quantities of circulating estrogens after aromatase inhibitor therapy to activate downstream pathways[447,454].

Moreover, mutations and translocations in the estrogen receptor gene, such as those that allow ligand-independent signaling, can induce aromatase inhibitor resistance[455]. Second, mutations in p53 can cause both *de novo* and acquired resistance to aromatase inhibitor therapy and is often associated with more aggressive ER+ disease[455]. Finally, activity of other signaling pathways, such as those mediated by EGFR and HER2, can induce resistance to aromatase inhibitors[454,455]. Importantly, cross-resistance between steroidal and non-steroidal aromatase inhibitors does not always occur. This allows for a trial of the alternative aromatase inhibitor class, should resistance to the initial class emerge[456].

The pharmacokinetics of the three aromatase inhibitors are similar. All three drugs are administered orally once per day and achieve maximal efficacy after 2-4 days for anastrazole and letrozole and 7 days for exemestane[457]. The elimination half-lives of letrozole, anastrazole, and exemestane are 42 hours, 41 hours, and 27 hours respectively[458–460]. All three aromatase inhibitors are metabolized by different CYP450 enzymes and undergo different excretion patterns. Letrozole is metabolized by CYP3A4 and CYP2A6 into an inactive metabolite, glucuronidated, and renally excreted[461]. In contrast, anastrazole undergoes hepatic metabolism through CYP1A2, CYP2C8/9, and CYP3A4, with primarily biliary excretion and minimal renal involvement[457,462]. Finally, exemestane is extensively metabolized by CYP3A4 and excreted equally in the bile and urine[463]. The primary toxicities of aromatase inhibitors are musculoskeletal, most commonly seen as osteoporosis and bone fracture[451,454]. Some studies have also suggested that aromatase inhibitors carry a significantly higher risk of ischemic cardiovascular events when compared to tamoxifen, but these differences are quite small numerically[454]. Beneficially, since aromatase inhibitors possess no estrogen agonist activity, they do not exhibit the increased risk of endometrial cancer

and thromboembolic disease that is seen with tamoxifen (see "Selective Estrogen Receptor Modulators" above)[443,448].

1.10.1.4 Luteinizing Hormone Releasing Hormone (LHRH) Analogues

Initial treatment for estrogen receptor positive breast cancer, prior to the era of tamoxifen and aromatase inhibitors, involved ovarian suppression, either by surgical castration or medical castration using luteinizing hormone releasing hormone (LHRH) analogues. The most commonly used LHRH analogue is goserelin, but leuprolide and triptorelin have also been studied. A large meta-analysis demonstrated that while LHRH usage with chemotherapy had no impact on breast cancer survival or recurrence in premenopausal breast cancer patients, women receiving LHRH analogues had a lower rate of premature ovarian failure[464]. These data suggest LHRH analogues might have use in preserving fertility in young women receiving chemotherapy. However, as these drugs are not commonly used in the treatment of breast cancer, their pharmacology will not be further discussed.

1.10.2 HER2 Targeted Therapies

HER2 is overexpressed in 15-30% of human breast cancers and is a marker for aggressive disease[169–171]. This has motivated the development of several therapies that target HER2, of which there are currently three different classes. Trastuzumab and trastuzumab emtansine (T-DM1) bind to the extracellular domain 4 of HER2, pertuzumab binds to the extracellular domain 2 of HER2, and lapatinib directly inhibits the kinase activity of both HER2 and EGFR[465].

1.10.2.1 Trastuzumab and T-DM1

Trastuzumab, also known as Herceptin, was approved in 1998 for the treatment of HER2 overexpressing breast cancer in both early and advanced disease. For early breast cancer, trastuzumab can be used in the adjuvant setting as combination therapy or as a single agent after completion of a multi-modality anthracycline-based therapy regimen[466]. For metastatic breast cancer, trastuzumab is used either in combination with paclitaxel or as a single agent in patients whom have received at least one prior chemotherapeutic regimen[466]. Due to its extended clinical use for the past twenty years, trastuzumab has become the standard of care for patients with HER2 positive breast cancer[467,468]. In 2013, trastuzumab conjugated with emtansine (T-DM1), a microtubule inhibitor, was approved for use in metastatic breast cancer patients previously treated with trastuzumab and a taxane[469].

Trastuzumab and T-DM1 work by similar mechanisms of action. First, when trastuzumab binds to the extracellular domain 4 of HER2, it inhibits formation of ligand-independent HER2-HER3 heterodimers, which form when HER2 is overexpressed[470]. Second, trastuzumab binding prevents the proteolytic cleavage and shedding of the HER2 extracellular domain, inhibiting formation of the kinase active membrane bound fragment p95[471]. Third, when trastuzumab binds to HER2, it induces antibody-dependent cellular cytotoxicity (ADCC) by engaging Fc receptors on effector immune cells[472]. Finally, it has been proposed that trastuzumab may induce HER2 internalization and degradation through activation of c-Cbl[473]. In addition to these mechanisms, T-DM1 possesses cytotoxic mechanisms of action due to the conjugated emtansine. Internalization of HER2 – T-DM1 complexes allows lysosomal degradation of MCC, a non-reducible thioether that links trastuzumab and emtansine, which releases emtansine into the cell[474]. Subsequently, emtansine binds to microtubules and inhibits their assembly, resulting in mitotic arrest or

catastrophe, and eventual cell death[474]. For additional information on the mechanisms of action of other anti-microtubule agents, see Section 1.9.3, “Mitotic Inhibitors”.

Resistance to trastuzumab arises through multiple mechanisms. First, polymorphisms in the Fc receptor on immune cells may decrease trastuzumab-mediated ADCC and result in worse prognosis[475]. Second, overexpression of other HER family members (in particular EGFR and HER3) and other tyrosine kinase receptors, such as insulin growth factor 1 receptor (IGF1R), are commonly implicated in trastuzumab resistance[476]. Third, alterations of HER2, including shedding of the extracellular domain or expression of splice variants, can decrease response to trastuzumab[477]. Finally, hyper-activation of the PI3K – Akt signaling pathway by multiple mechanisms has been shown to cause trastuzumab resistance[476]. The mechanisms of resistance to T-DM1 are not well understood but are likely associated with decreased HER2 expression, shedding of the extracellular domain, altered receptor-complex internalization, or drug efflux by MDR1[474].

Both trastuzumab and T-DM1 are administered by intravenous infusion. While trastuzumab has a relatively long elimination half-life of 28 days, T-DM1 has a shorter half-life of 4 days due to proteolytic degradation of the emtansine linker[466,478,479]. The elimination pathways of trastuzumab and T-DM1 are unknown but are hypothesized to be similar to homeostatic management of immunoglobulin G[466]. The most clinically significant toxicities associated with trastuzumab treatment are cardiotoxicity and pulmonary toxicity, which manifest most aggressively as congestive heart failure and pneumonitis respectively[466,480]. The most clinically significant toxicities associated with T-DM1 use are thrombocytopenia and transaminitis (the elevation of AST and ALT), without any reports of hepatic injury[481].

1.10.2.2 Pertuzumab

While trastuzumab is the standard of care for women with HER2 positive breast cancer, approximately 15% of women relapse despite trastuzumab therapy[482]. Pertuzumab was approved in 2012 for women with HER2 positive breast cancer in combination with trastuzumab and chemotherapy[483,484]. Pertuzumab binds to the extracellular domain 2 of HER2 and is effective at reducing ligand-dependent formation of HER2 heterodimers, most specifically heregulin-induced HER2-HER3 dimers[485,486]. In contrast, trastuzumab is only effective at blocking ligand-independent dimerization[470]. Similar to trastuzumab, the Fc domain of pertuzumab can interact with the Fc receptor on immune cells and induce ADCC[487]. Resistance mechanisms to pertuzumab are not as well characterized as those of trastuzumab. Some proposed resistance mechanisms include formation of HER3-EGFR heterodimers, HER2 extracellular domain shedding, and PIK3CA expression[488,489].

Pertuzumab is administered by intravenous injection and has a terminal elimination half-life of approximately 19 days[490]. Similar to trastuzumab, the elimination of pertuzumab is thought to be conducted similarly to homeostatic management of immunoglobulin G[490]. The most clinically severe toxicities observed are cardiotoxicity, most commonly a decrease in left ventricular ejection fraction (LVEF). Importantly, LVEF dysfunction incidence is not significantly increased when pertuzumab is added to trastuzumab therapy[491].

1.10.2.3 Lapatinib

Lapatinib is a dual specificity EGFR/HER2 small molecule kinase inhibitor. It is currently approved for women with HER2 positive breast cancer in combination with capecitabine who have received prior therapy with trastuzumab, an anthracycline, and a taxane[492]. Additionally, lapatinib, in combination with letrozole, is approved for use in hormone receptor positive metastatic breast

cancer that overexpresses HER2[492]. The mechanism of action of lapatinib is inhibition of EGFR and HER2 kinase activity by acting as an analogue of ATP to prevent ATP-mediated transphosphorylation of receptor dimers[493]. Additionally, lapatinib can also impact HER3 phosphorylation in the context of EGFR-HER3 or HER2-HER3 heterodimers[494]. Disruption of these receptor tyrosine kinases inhibits downstream signaling through MAPK, PI3K-Akt, and PLCγ[494]. Resistance to lapatinib most commonly occurs with activation of compensatory pathways, including other receptor tyrosine kinases and intracellular kinases, or mutation of the tyrosine kinase domain of HER2[495].

Lapatinib is an orally administered agent and exhibits an elimination half-life of 24 hours[493]. Lapatinib is primarily eliminated by biliary excretion after hepatic metabolism by CYP3A4[496]. Importantly, lapatinib metabolites can act as covalent inhibitors of CYP3A4, which can cause drug-drug interactions and hepatotoxicity[497,498]. Lapatinib is generally well-tolerated, with the most common adverse effects being diarrhea, rash, nausea, and fatigue[499].

1.10.3 EGFR Targeted Therapies

EGFR targeted therapies have been successfully employed in colorectal, lung, and head and neck cancer since the initial approval of gefitinib for non-small cell lung carcinoma in 2003[500]. Unfortunately, the response of breast cancers to EGFR inhibitors has been mixed – while some patients respond to therapy, clinical trials have shown little benefit, likely due to insufficient patient stratification[501,502]. Clinical trials to determine whether EGFR inhibitors can provide survival benefit in breast cancer, in particular triple negative breast cancers which often overexpress EGFR, are currently being conducted. As there are no approved EGFR inhibitors for

breast cancer (except for lapatinib, discussed above in "HER2 Targeted Therapies"), the pharmacology of this drug class will not be further discussed.

1.10.4 CDK4/6 Targeted Therapies

Cyclin dependent kinase 4/6 (CDK4/6) inhibitors were developed to help overcome the endocrine therapy resistance that is often seen with advanced hormone receptor positive breast cancer[503]. Additionally, CDK4/6 selective inhibitors were developed due to the high incidence of dose-limiting toxicities with pan-CDK inhibitors[503]. There are three CDK4/6 inhibitors currently approved for the treatment of advanced hormone receptor positive, HER2 negative, advanced breast cancer: palbociclib, ribociclib, and abemaciblib[503,504]. These drugs are currently in clinical trials to extend their application to different breast cancer subtypes, in particular triple negative breast cancer[503,505].

The mechanism of action of CDK4/6 inhibitors is to block the G1 to S phase transition in the cell cycle, resulting in growth arrest[506]. This checkpoint, also known as the restriction or R point, is mediated by a complex formed between cyclin D and either CDK4 or CDK6. This complex hypophosphorylates retinoblastoma (Rb), which then allows the complex of Cyclin E and CDK2 to hyperphosphorylate Rb upon further passage through the R point. Hyperphosphorylation of Rb results in the release of bound E2F, which subsequently activates S phase genes that mediate further cell cycle progression[506]. Importantly, inhibition of CDK4 and CDK6 is both necessary and sufficient to induce cell cycle arrest, whereas the role of CDK2 is less essential for progression through the R point[507].

There are many mechanisms of CDK4/6 inhibitors, all of which can either result in intrinsic or acquired resistance. Since CDK4/6 inhibitors act by preventing the phosphorylation of Rb, only

tumors with functional Rb, which account for 65-80% of all and 97% of ER+/HER2- breast cancers, are treatment responsive[506,508,509]. Moreover, tumors that express high levels of $p16^{Ink4a}$ (an endogenous CDK4/6 inhibitor) are often resistant to CDK4/6 inhibitors. Additionally, 3-phosphoinositide-dependent protein kinase 1 (PDK1) expression has been shown to decrease sensitivity to CDK4/6 inhibition[510]. Finally, overexpression of Cyclin E may result in resistance to CDK4/6 inhibitors, but no clinical association has been shown between Cyclin E amplification and CDK4/6 inhibitor response[509,511,512].

Attractively, all three clinically approved CDK4/6 inhibitors are orally administered medications that exhibit slow absorption kinetics[503]. These inhibitors are extensively tissue bound, metabolized, and primarily eliminated through the biliary system with elimination half-lives of 26 hours, 36 hours, and 17-38 hours for palbociclib, ribociclib, and abemaciclib respectively[513–515]. Drug-related adverse events are relatively common with this drug class due to the ubiquity of the cell cycle. While the most common dose-limiting toxicity with palbociclib and ribociclib is neutropenia, the most common dose-limiting toxicity with abemaciclib is fatigue[503]. The lower incidence of neutropenia observed with abemaciclib may be related to its relatively higher selectively for CDK4 than CDK6[514].

1.10.5 PARP Targeted Therapies

Poly(ADP-ribose) polymerases (PARPs) are a family of enzymes that play a role in the cellular response to DNA damage. The ADP-ribose polymer that is synthesized by PARP enzymes targets the assembly of the DNA repair complex at DNA damage sites[516]. Currently, there is only one FDA approved PARP inhibitor for breast cancer therapy. Olaparib is indicated for treating metastatic breast cancer in patients who possess a germline BRCA1/2 mutation. While olaparib

demonstrated a 3 month increase in progression free survival and improvement in patient quality of life, there was no overall survival benefit[517]. Many clinical trials are underway to extend PARP inhibitor usage to breast cancer patients without a germline BRCA1/2 mutation, in particular in the context of triple negative breast cancer[518]. Additionally, other PARP inhibitors, such as veliparib, niraparib, and talazoparib, are being investigated for their efficacy[516]. However, since PARP inhibitors are not employed that frequently for the treatment of breast cancer, their pharmacology will not be further discussed.

1.10.6 PD-1 and PD-L1 Targeted Therapies

Immune checkpoint blockade through targeting PD-1 or its cognate ligand PD-L1 has proven quite successful in metastatic melanoma, non-small cell lung cancer, Hodgkin's lymphoma, and head and neck cancer[519]. Currently, clinical trials are underway to investigate pembrolizumab (PD-1 inhibitor) and atezolizumab (PD-L1) in the context of breast cancer. The majority of these studies, including the two phase 3 clinical trials, test PD-1/PD-L1 inhibition efficacy in advanced triple negative breast cancer, which is thought to be more susceptible to checkpoint blockade due to the higher level of tumor infiltrating lymphocytes (TILs) found in this breast cancer subtype[520]. However, as there are currently no immune checkpoint blockade agents approved for the treatment of breast cancer, their pharmacology will not be further discussed.

1.11 COMMON SYSTEMIC THERAPY REGIMENS

The systemic therapy regimens for breast cancer vary based on cancer molecular subtype and stage. This section will briefly describe the preferred regimens based on the national comprehensive cancer network (NCCN) guidelines[266]. For early breast cancer (stage I and II), chemotherapy is often given in the adjuvant setting, after surgery and prior to radiotherapy[266]. Common regimens for triple negative breast cancer are 1) dose-dense doxorubicin and cyclophosphamide followed by paclitaxel and 2) combination docetaxel and cyclophosphamide. Common regimens for HER2 positive disease are 1) doxorubicin and cyclophosphamide followed by paclitaxel and trastuzumab and 2) trastuzumab, docetaxel, and carboplatin, with or without pertuzumab. Estrogen receptor positive disease is treated in the adjuvant setting with five years of tamoxifen or an aromatase inhibitor, for pre- and postmenopausal women respectively[521]. Some early stage estrogen receptor positive breast cancers are treated with chemotherapy, especially for large primary tumors or node positive disease[522]. For locally advanced breast cancer (Stage III), chemotherapy is given in the neoadjuvant setting to increase the success rate of the surgical intervention[523]. Additional adjuvant chemotherapy is rarely used in locally advanced breast cancer, but adjuvant radiotherapy plays an important role in management[523,524].

For metastatic breast cancer (Stage IV), systemic therapy is the primary form of treatment[266]. Estrogen receptor positive disease is treated with tamoxifen or an aromatase inhibitor, for premenopausal and postmenopausal women respectively. Postmenopausal women commonly receive a CDK4/6 inhibitor in combination with an aromatase inhibitor to increase progression free survival[525]. HER2 positive metastatic breast cancer is often treated with a taxane (paclitaxel or docetaxel) in combination with trastuzumab and pertuzumab[266]. Resistant disease is commonly treated with T-DM1, lapatinib with capecitabine, or trastuzumab combined with vinorelbine or

capecitabine[266]. Triple negative breast cancers can be treated with a multitude of single agent or combination therapies, such as gemcitabine, eribulin, gemcitabine with carboplatin, or cyclophosphamide with methotrexate and 5-FU[266].

In summary, the management of stage I and II breast cancer commonly involves chemotherapy in the adjuvant setting. Estrogen receptor positive disease is often treated with tamoxifen or an aromatase inhibitor, with chemotherapy added for locally aggressive disease. HER2 positive disease is treated with chemotherapy and trastuzumab. Triple negative breast cancer is treated with combination chemotherapy. Stage III breast cancer, in contrast, employs neoadjuvant chemotherapy instead of adjuvant chemotherapy in order to improve surgical success rates. Finally, the treatment of metastatic breast cancer is complex, multifactorial, and depends on the molecular subtype of the breast cancer, previous treatment modalities, and patient and doctor preferences[526].

1.12 CLINICAL NEED

To enhance the current standard of care, there exists a great clinical need for drugs that can delay or eliminate the mortality from metastatic breast cancer. This need is best illustrated in the case of estrogen receptor positive breast cancer patients, many of whom may demonstrate an apparent cure of their disease for decades before dormant micrometastases outgrow into clinically evident macrometastases. Once metastatic disease is apparent, treatment is challenging and often palliative rather than curative. Thus, one approach to limit the burden of metastatic breast cancer would be to maintain dormant metastatic tumor cells in a quiescent state, delaying this mortal phase of metastatic outgrowth and recurrence.

Clinical development and implementation of new drugs takes years to decades of study and is very costly. As such, repurposing already FDA-approved drugs with favorable safety profiles may allow for more rapid clinical implementation of effective therapies at low cost[527,528]. In particular, drugs that are employed in the treatment of chronic health conditions with few or mild adverse events would be the most suitable for use as a long term adjuvant therapy to prevent emergence of dormant micrometastases.

The statin drugs have been clinically used for the treatment of cardiovascular disease for three decades and are well tolerated by the majority of patients[529,530]. Large retrospective, population-based studies have shown that statins reduce breast cancer mortality[531–533] without influencing incidence of the primary tumor[534–536]. These clinical data have been supported by cell and animal studies that suggest statins suppress growth, induce apoptosis, and/or decrease invasiveness of breast cancer cells[537–542]. However, it remains unclear whether statins can suppress outgrowth of metastases in the context of the metastatic microenvironment and what factors guide statin efficacy in cancer. The next section will present the current usage and pharmacology of the statin drug class as well as discuss basic science and clinical studies that investigate the effects of statins on cancer.

2.0 STATIN PHARMACOLOGY

2.1 DISCOVERY

The discovery and development of the statin drugs was motivated by incontrovertible evidence from the Framingham Heart Study that high blood cholesterol levels increased the risk for future myocardial infarction[543]. These epidemiological studies drove investigations into the biochemical synthesis of cholesterol and its physiologic regulation[544]. Subsequent studies demonstrated that dietary cholesterol could reduce the synthesis of mevalonate, while the synthesis of its precursor, β-Hydroxy β-methylglutaryl-CoA (HMG-CoA), was unaffected[545,546]. These data suggested that the enzyme responsible for this conversion, HMG-CoA reductase (HMGCR), is an important node for the regulation of *de novo* cholesterol synthesis[546]. In parallel, other cholesterol-lowering agents, such as cholesterol excreting agents and fibrates, were found to have limited success due to insufficient efficacy or safety concerns[547]. Moreover, the first clinically used drug that inhibited cholesterol biosynthesis, triparanol, was withdrawn from the market for severe ocular and cutaneous adverse events[548–551].

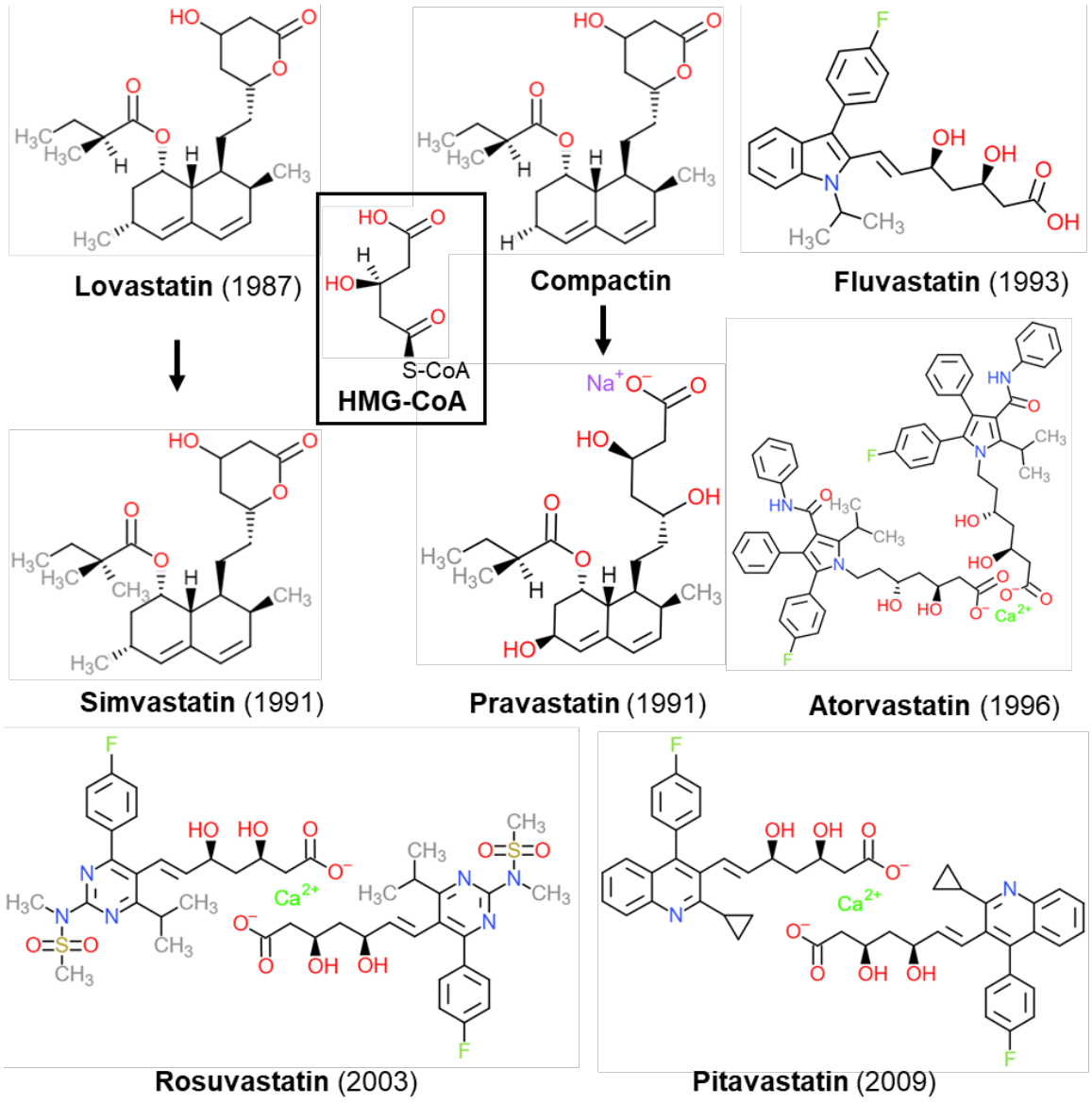


Figure 7. Chemical structures of HMG-CoA, compactin, and FDA-approved statins

The chemical structures of HMG-CoA, compactin, the first derived statin, and the seven FDA-approved statin drugs. Dates in parentheses indicate the initial approval date by the FDA, as according to their website[552]. Drug structures were drawn using ChemDoodle Sketcher[553].

The first HMGCR competitive inhibitors were isolated by Akira Endo, who hypothesized that fungal compounds could inhibit HMGCR activity[547]. Two compounds with potent HMGCR inhibitory activity were isolated in the 1970s, named citrinin and compactin (also known as mevastatin)[547,554,555]. Dr. Endo noted that the pharmacophore of compactin is strikingly similar to the structure of HMG-CoA (Figure 10). Unfortunately, citrinin and compactin never reached clinical approval due to concerns about renal toxicity and carcinogenicity, respectively[547]. Several years later, in 1979, Dr. Endo and Merck Research Laboratories independently isolated the same statin from fungal cultures, which was eventually named lovastatin[547]. After promising clinical studies that demonstrated dramatic reductions in blood cholesterol and no carcinogenicity, lovastatin became the first FDA approved statin in 1987[547,556].

Since the approval of lovastatin, seven other statins have garnered FDA approval: atorvastatin, cerivastatin, fluvastatin, pitavastatin, pravastatin, rosuvastatin, and simvastatin (Figure 10) [529,547,557]. Cerivastatin was subsequently withdrawn from the market in 2001 due to significant mortality from rhabdomyolysis-induced kidney failure[557]. While atorvastatin, fluvastatin, pitavastatin, and rosuvastatin are synthetic, pravastatin and simvastatin are semisynthetic, being derived with minor modification from compactin and lovastatin respectively[547].

2.2 PHARMACODYNAMICS

All statin drugs are competitive inhibitors of the enzyme HMGCR. Statins bind to a portion of the HMGCR catalytic domain, which inhibits binding of the natural substrate HMG-CoA[530,558]. Statins

bind the active site of HMGCR with a 100-1000 fold higher affinity than HMG-CoA[559]. Importantly, while all statins share the same pharmacophore (Figure 10), the structural characteristics guide their binding affinity to HMGCR[560]. The mean inhibitory concentration (K_i) of the FDA approved statins and the affinity of HMG-CoA for HMGCR are given in Table 14[559,561,562].

Table 11. Affinity of HMG-CoA and statins for HMGCR

Molecule	HMG-CoA	Atorva	Fluva	Lova	Pitava	Prava	Rosuva	Simva
K_d or K_i (nM)	6600	15.2	17.9	2.7 – 11.1	6.8	55.1	12	18.1

The affinity (K_d) or mean inhibitory concentration (K_i) of HMG-CoA and statins, respectively, for HMGCR. The statin prefixes are used instead of the whole name (e.g. Atorva = Atorvastatin). Affinity and mean inhibitory concentration values are reported in nanomolar (nM)[559,561,562].

Statins dramatically reduce flux through the cholesterol biosynthesis pathway by their inhibition of the rate limiting enzymatic step, catalyzed by HMGCR[563]. The most significant steps and branch points of the cholesterol biosynthesis pathway are shown in Figure 11. The most biologically relevant molecules affected by inhibition of HMGCR are downstream of mevalonate – cholesterol, farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP). The mechanisms of action of statin therapy, both in cardiovascular disease and other diseases, are related to reductions in each of these three compounds. Before describing how statins affect cancer growth (Section 2.6, “Statin Usage in Cancer”), it is important to first describe the mechanisms of action by which statins lower cardiovascular disease risk and mortality.

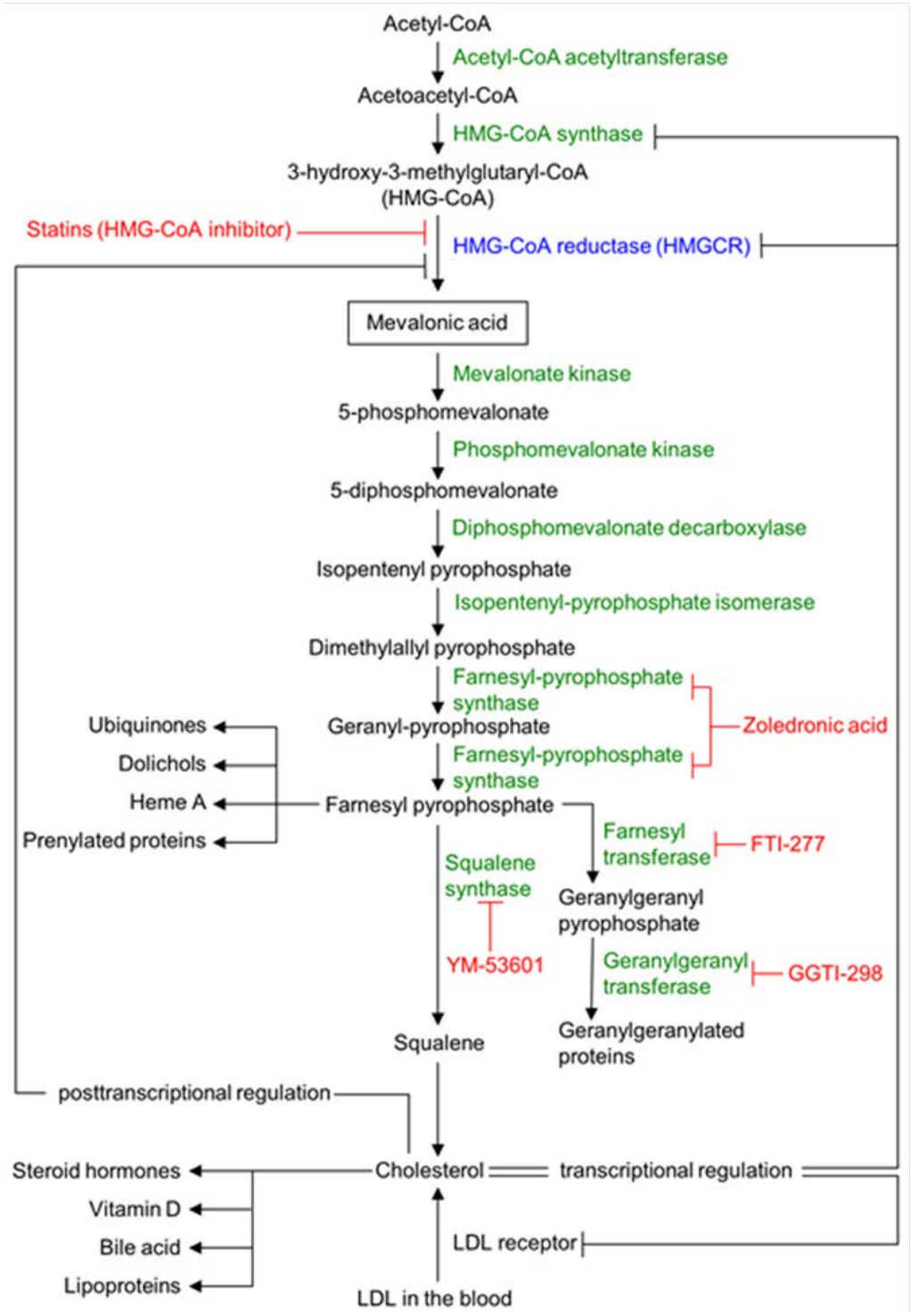


Figure 8. The cholesterol biosynthesis pathway

Statins block HMG-CoA reductase (HMGCR, shown in blue) to shut down the cholesterol biosynthetic pathway. Other inhibitors of this pathway are shown in red. In addition to cholesterol, other downstream mediators are affected, such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate.

2.2.1 Effects of Statins on Lipid Homeostasis

One of the main mechanisms of action of statin therapy is the reduction in circulating lipids, most specifically cholesterol in the form of low density lipoprotein (LDL) particles[564]. Statins accomplish this by acting selectively in the liver, the primary site of cholesterol biosynthesis[565], largely due in part to first pass metabolism. Statins enter hepatocytes by diffusion or active transport (see "Pharmacokinetics")[530]. Statin binding to HMGCR prevents it from converting HMG-CoA into mevalonate, which reduces the intracellular cholesterol concentration[566]. When cells are deprived of cholesterol, the transport protein SREBP cleavage-activating protein (SCAP) shuttles sterol regulatory element-binding protein 2 (SREBP-2) from the ER to the Golgi, where Site-1 Protease (S1P) and Site-2 Protease (S2P) reside[567]. These two proteases subsequently cleave SREBP-2 into its N-terminal active form, which translocates into the nucleus and binds sterol response elements (SREs) to enhance transcription of several target genes, including HMGCR, Fatty Acid Synthase (FAS), LDL Receptor (LDLR), and Proprotein Convertase Subtiliskin/Kexin Type 9 (PCSK9)[567–569]. Increased expression of LDLR facilitates uptake of LDL particles into hepatocytes, which reduces blood cholesterol[570]. Conversely, PCSK9 acts as a negative regulator of LDLR and is negatively regulated by circulating LDL[569,571,572]. In short, statins both reduce new cholesterol synthesis and enhance hepatic uptake of existing, circulating cholesterol. Moreover, statin-mediated hepatic uptake of LDL particles also reduces circulating triglyceride levels in patients with hypertriglyceridemia[573].

Statin treatment also affects other aspects of lipid homeostasis. Even in patients with dysfunctional LDLRs, statins decrease LDL by inhibiting synthesis of apolipoprotein B100, an essential protein for the formation of LDL particles[574]. Additionally, statins enhance circulating levels of high density lipoprotein (HDL), which plays an important role in removing cholesterol

from peripheral tissues and returning it to the liver[575]. The increase in HDL is thought to be due to inhibition of Cholesterol Ester Transfer Protein (CETP) function, which normally acts to return cholesterol esters to LDL particles[575,576]. Finally, statins can lessen the oxidation of LDL particles and decrease their uptake by macrophages through regulation of scavenger receptor expression[577].

2.2.2 Effects on Cardiovascular Pathobiology

In addition to modifying lipid homeostasis, statins exert pleotropic effects on other aspects of cardiovascular pathobiology. Cell-mediated effects stem from reductions in cellular signaling from small G-proteins through reducing the availability of FPP and GGPP[564]. In endothelial cells, decreased activation of Rho enhances expression of endothelial nitric oxide synthase (eNOS), which protects endothelial function and impedes cardiovascular disease progression[578]. In smooth muscle cells, reduction of Ras and Rho signaling reduces their proliferation and migration, which decreases the intimal hyperplasia associated with vascular injury[579,580]. In inflammatory cells, statins act to decrease their proliferation and secretion of proinflammatory cytokines, such as IL-6, IL-8, and MCP-1[581,582]. Finally, in myocardial cells, statins can reduce cardiac remodeling through suppression of Ras and Rac1[583].

In comparison, additional benefits of statins in cardiovascular disease involve inhibition of disease progression. Acute coronary syndromes are the result of rupture of an unstable plaque in the vessel wall, which induces thrombosis and subsequent occlusion of blood flow. Statins can modify the composition of plaques, reducing the concentration of lipids, increasing collagen deposition, and decreasing metalloproteinase activity[584]. Statins also impede thrombosis by inhibiting platelet activation and the coagulation cascade, in part through reductions in Na+/H+

antiporter activity and tissue factor expression respectively[585,586]. Finally, statins can facilitate dissolution of thromboses by upregulation of tissue plasminogen activator (tPA)[587].

2.3 PHARMACOKINETICS

The seven FDA approved statins have similar, but not identical, pharmacokinetics. Pharmacokinetic properties of the seven FDA approved statins are summarized in Table 15, and the salient differences between the different drugs are discussed below. Statins are classified as lipophilic or hydrophilic depending on their partition coefficients. The value of the partition coefficient is dependent on pH, which controls the ionization state of the drug[588]. The partition coefficients reported in Table 15 were obtained at pH 7.4 (defined as log D), which are lower than other reported values in the literature that were obtained at acidic pH values (pH = 2.0)[588]. Accordingly, the hydrophilic statins pravastatin and rosuvastatin have Log D values less than zero, whereas the other five statins have Log D values greater than zero and are classified as lipophilic statins[589].

All statins are administered orally, with relatively little of the administered dose reaching the systemic circulation. The highest orally bioavailable statin is pitavastatin (51%), whereas the other six statins exhibit an absolute bioavailability less than 25% (Table 15)[589]. All statin drugs reach peak blood concentration levels one to four hours after administration. All statins are extensively protein bound in the serum, except pravastatin, and as a result, exhibit minimal extra-hepatic tissue distribution[589,590]. After systemic absorption, statins undergo first pass metabolism in the liver. Simvastatin and lovastatin are administered as lactone prodrugs, which must be hydrolyzed in the gut and liver to generate the active statin acid[589].

Table 12. Pharmacokinetic properties of the seven FDA approved statins

Property	Atorva	Fluva	Lova	Pitava	Prava	Rosuva	Simva
Log D	1.11	1.27	1.7	1.49	-0.84	-0.33	1.60
Bioavailability (%)	14	24	<5	51	17	20	<5
T_{max} (h)	1-2	<1	2-4	1	1-1.5	3-5	4
Protein Binding (%)	> 98	98	>95	99	50	88	95
CYP450 Isozyme	3A4	2C9	3A4	2C9 2C8	None	2C9	3A4
Active Metabolites (n)	Yes (2)	No	Yes (4)	No	No	Minimal	Yes (3)
Elimination $T_{1/2}$ (h)	14	1.2	3	11	1.8	19	2
Renal Excretion (%)	<2	<6	10	15	20	10	13

Absorption, distribution, metabolism, and excretion parameters for each statin are listed. The statin prefixes are used instead of the whole name (e.g. Atorva = Atorvastatin). Times are represented in hours (h). Log D = distribution coefficient at pH 7.4 (higher value means more lipophilic); T_{max} = time after oral dose to maximum serum concentration; CYP450 = cytochrome P450; $T_{1/2}$ = half-life. Data were acquired from Schachter 2005[530], McKenney et al 2009[589], and Davidson et al 2006[591].

Statin uptake into cells can occur through passive diffusion (limited to lipophilic statins) or carrier-mediated transport, most commonly by OATP1B1 with lesser contributions from OATP1B3, 1A2, and 2B1[592,593]. OATP isoforms 1B1 and 1B3 are exclusively expressed on the sinusoidal membrane of hepatocytes, whereas isoforms 1A2 and 2B1 are expressed on other cell types, such as enterocytes, neurons, and myocytes. Transporter polymorphisms, particularly in OATP1B1, can increase plasma concentrations of statins and increase the risk for toxicity[592–595]. OATP2B1 has been shown to be an uptake receptor for atorvastatin and rosuvastatin in skeletal muscle fibers, suggesting it plays a role in statin myotoxicity[596]. Importantly, except for hepatocytes, the majority of cell types do not express these transporters at meaningful levels[592].

This means lipophilic statins can distribute into extrahepatic tissues by diffusion across the cell membrane, whereas hydrophilic statins are inable[530].

Statins undergo differential metabolism by the CYP450 isozymes. Atorvastatin, lovastatin, and simvastatin are extensively metabolized by CYP3A4 into pharmacologically active metabolites (Table 15)[589]. In contrast, fluvastatin is extensively metabolized by CYP2C9, while pitavastatin and rosuvastatin are minimally (10%) metabolized[589]. Pravastatin exhibits no metabolism by the CYP450 family members. Statins are primarily eliminated by biliary excretion, with terminal elimination half-life values between 1.2 and 19 hours (Table 15)[530]. Efflux of statins and their metabolites from cells is primarily accomplished by the transporter ABCB1, also known as multi-drug resistance 1 (MDR1), and polymorphisms in the gene that encodes this transporter can increase the risk for statin myotoxicity[597].

2.4 TOXICITY

Statins are typically well tolerated drugs. The most common adverse events associated with statin usage involve muscle tissues, which range in severity from myalgia to fatal rhabdomyolysis[530]. Muscle pain (myalgia) is relatively more common among statin users, with an incidence of approximately 5%[598]. Myopathy, or myositis, only occurs in 0.1% of patients taking statins and involves pain, weakness, and mobility restrictions that often are accompanied by an elevation in serum creatine kinase (CK) to ten times the upper limit of normal (ULN)[598]. Rarely, myopathy can progress to rhabdomyolysis, which involves CK elevations above fifty times the ULN and myoglobin accumulation in the blood (myoglobinemia) and urine (myoglobinuria), which can induce renal failure[598]. The incidence of rhabdomyolysis is extremely rare, with an incidence of

3.4 per 100,000 person years and mortality rate of approximately 10%[599]. As stated previously, cerivastatin was removed from the market in 2001 because it exhibited a rhabdomyolysis incidence of approximately 46 per 100,000 person years[557,599]. Rhabdomyolysis incidence is higher in patients taking atorvastatin, lovastatin, and simvastatin, which is due to their extensive metabolism by CYP3A4 and potential for drug-drug interactions[600].

The less common adverse events observed with statin usage involve the liver, pancreas, kidney, and peripheral nerves. The most common presentation of hepatotoxicity is an asymptomatic, often transient, elevation in serum transaminases, which occurs in 0.5-3% of patients[589,600,601]. Serious liver injury and liver failure, as a result of statin therapy, is extremely rare and similar to the rates in the population not taking statins[589]. Statins have been shown to increase the risk of diabetes by approximately 9%, which may be due to the influence of cholesterol on pancreatic β-cell function[589,602]. Statins have also been associated with proteinuria and acute renal failure, the latter most often in the context of rhabdomyolysis (see above)[589,600]. Finally, statins have been associated with the development of peripheral neuropathy in some patients, which improves or disappears upon cessation of the statin[589,603].

2.5 CLINICAL USAGE

Statin usage in the United States has increased dramatically over the past several decades. Between 2003 and 2012, statin usage among adults in the US increased from 18% to 26%[604]. Any form of cholesterol lowering medication is taken by 71% of adults with cardiovascular disease, 63% of adults with diabetes, and 54% of adults with hypercholesterolemia[604]. Of these, statins are taken by 93% of patients receiving cholesterol lowering therapy[604]. The most commonly used statins in

2012 were simvastatin (42%), atorvastatin (20.2%), pravastatin (11.2%), rosuvastatin (8.2%), and lovastatin (7.4%)[604]. In parallel with cardiovascular disease risk, statin use in the US increases with age, with 17% of adults aged 40-59 and 48% of adults aged 75 and over using cholesterol lowering medication[604,605].

The clinical potency of statin drugs and their different dosage amounts is determined by the measurement of blood LDL cholesterol (LDL-C) in mg/dL, which has largely replaced total cholesterol as a measurement of cardiovascular risk[606]. Statin regimens that achieve <30%, 30-50%, or >50% reductions in LDL-C are labeled low-, moderate-, and high-intensity statin therapy respectively[607]. The seven statins and their approved daily dosages are organized by statin therapy intensity category below, in Table 16[607].

Table 13. High- Moderate- and Low-Intensity Statin Therapy

Low-Intensity	**Moderate-Intensity**	**High-Intensity**
Daily dose lowers LDL-C by **<30%**	Daily dose lowers LDL-C by **30-50%**	Daily dose lowers LDL-C by **>50%**
Fluvastatin 20-40mg	**Atorvastatin 10** *(20)* **mg**	**Atorvastatin 40-80mg**
Lovastatin 20mg	**Fluvastatin 40mg, BID**	**Rosuvastatin 20** *(40)* **mg**
Pitavastatin 1mg	*Fluvastatin XL 80mg*	
Pravastatin 10-20mg	**Lovastatin 40mg**	
Simvastatin 10mg	*Pitavastatin 2-4mg*	
	Pravastatin 40 *(80)* **mg**	
	Simvastatin 20-40mg	
	Rosuvastatin *(5)* **10mg**	

Statin dosages that achieve plasma reductions in LDL-C of <30% (Low-Intensity), between 30-50% (Moderate-Intensity), and >50% (High-Intensity), as tested in randomized control trials (RCTs) and reported in the 2013 American College of Cardiology (ACC) and American Heart Association (AHA) 2013 guidelines[607]. Statin dosages reported in *italics* are FDA approved but have not been tested in RCTs. BID = twice daily; XL = extended release

Statins are the first line treatment for hyperlipidemia and atherosclerotic cardiovascular disease (ASCVD) after therapeutic lifestyle changes and are also used as preventative therapy for patients at a high risk for developing cardiovascular disease[608]. The individuals that benefit from statin therapy in the context of ASCVD are patients with 1) clinical ASCVD, 2) primary elevations of LDL-C greater than 190 mg/dL, 3) diabetes, aged 40-75, with LDL-C between 70-189 mg/dL, and without clinical ASCVD, and 4) LDL-C between 70-189 mg/dL and estimated 10-year ASCVD risk greater than 7.5%[607]. Clinical ASCVD is defined as patients with a history of acute coronary syndromes, past myocardial infarction, stable or unstable angina, coronary or other arterial revascularization, stroke, transient ischemic attack, or peripheral artery disease[607]. Currently, moderate- and high-intensity statin therapy are recommended for the treatment of these patient groups, as they do not show an increased risk of statin-related adverse events over low-intensity therapy and provide additional ASCVD risk reduction[607].

2.6 STATIN USAGE IN CANCER

Statins have a significant impact on cardiovascular disease mortality without a high incidence of severe adverse events. In addition to lowering blood cholesterol, statins derive part of their cardiovascular mortality benefit from pleiotropic effects on the endothelium, smooth muscle, inflammatory cells, and myocardium, which are related to a reduction in prenylating intermediates that normally modify and activate critical signaling proteins involved with intracellular signaling[583]. These pleiotropic effects of statins have been investigated in the context of many other diseases. Of relevance to present work are the clinical and basic research studies that have investigated the roles of statin drugs in cancer incidence, progression, and mortality.

2.6.1 Clinical Research Studies

While few prospective clinical trials have been conducted to investigate statin therapy in cancer patients, multiple retrospective cohort studies and meta-analyses have been published, some with large patient numbers. There are several hypotheses that motivate these studies. First, since statins lower LDL-C, this many influence carcinogenesis and tumor progression, as rapidly dividing cells require increased cholesterol for cell membrane synthesis[609]. Conversely, low cholesterol has been associated with higher incidence of certain cancers[610]. Moreover, in the context of prostate cancer, cholesterol is an essential precursor for steroid hormone synthesis, which is involved with carcinogenesis and disease progression[609,611]. Similarly, statins can compete with dehydroepiandosterone sulfate (DHEAS) for OATP2B1-mediated uptake into prostate cancer cells, thereby reducing the concentration of testosterone precursors[612,613]. In addition, the influence of statins on reducing prenylation, discussed above in the context of cardiovascular disease, may reduce signaling through pathways involved in carcinogenesis and cancer progression, such as Ras and Rac1[583,614]. Finally, statins reduce the secretion of pro-inflammatory cytokines by immune cells, which have been shown to drive carcinogenesis and metastasis[615,616].

Since these three factors influence both early (carcinogenesis) and late (metastatic cascade) stages of cancer progression, clinical studies have investigated the influence of statins on cancer incidence, recurrence, and mortality. These studies will be discussed in the following three sections below in the context of these categories, and the data are summarized in Table 17. While data on many cancer types will be presented, the primary cancer types discussed will be prostate cancer and breast cancer. Prostate cancer will be discussed because it is the most common cancer in men, and steroid hormone synthesis is mechanistically affected by statins[611]. Breast cancer will be the

primary focus of these three sections because the objective of this thesis is to investigate the role of statins in preventing the emergence of dormant metastatic breast cancer.

Table 14. The Influence of Statin Therapy on Cancer Incidence, Recurrence, and Mortality

Cancer Type	The Effect of Statin Therapy on Cancer…		
	Incidence	**Recurrence**	**Mortality**
Breast	↔	↔ (Hydrophilic Statins) ↓ (All or Lipophilic Statins)	↔ (Hydrophilic Statins) ↓ (All or Lipophilic Statins)
Prostate	↔	↔ (Radical Prostatectomy) ↓ (Radiotherapy)	↓ (All, Lipophilic, or Hydrophilic Statins)
Lung	↔	↔ (Pravastatin) ↓ (All Statins)	↔ (Pravastatin) ↓ (All Statins)
Colorectal	↔	↓ (All Statins)	↓ (All Statins)
Primary Liver	↓ (All Statins)	↓ (All Statins or Pravastatin)	↓ (All Statins or Pravastatin)

The influence of statins on cancer incidence, recurrence, and mortality for breast, prostate, lung, colorectal, and primary liver cancer. ↔ = no effect, ↓ = reduction. All statins, hydrophilic statins, and lipophilic statins refer to studies that were conducted examining patients taking any statin, any hydrophilic statin, or any lipophilic statin respectively.

2.6.1.1 Statin Influence on Cancer Incidence

Many studies have investigated the influence of statin therapy on the risk for developing cancer, with conflicting results. The CARE trial in 1996 published one of the first associations between statins and cancer incidence, in which pravastatin users demonstrated a slight increase in the risk of many types of cancer and a statistically significant increase in breast cancer risk[617]. Similarly, the PROSPER trial in 2002 showed a significant increase in new cancer diagnoses in patients receiving pravastatin than those on placebo[618].

In contrast, some studies demonstrate a decreased risk of developing cancer while taking statins. For example, Poynter and colleagues found that patients taking simvastatin or pravastatin were at a lower risk for developing colorectal cancer[619]. However, decreased cholesterol is an independent risk factor for developing colorectal cancer, and other research has shown that cancer risk is unaffected in statin users that continue or discontinue statin therapy[620]. Shi and colleagues demonstrated a reduction in the risk of liver cancer among users of any statin drug[621]. Other studies have corroborated the risk reduction benefit of statins on liver cancer, which could be explained by the relatively higher local concentrations of statins in the liver due to drug distribution[622,623].

The largest studies focus on colorectal, lung, prostate, and breast cancer[535,624–629]. In colorectal cancer, three large retrospective cohort studies demonstrated no influence of statins on disease incidence[630–632]. In lung cancer, the largest cohort and meta-analysis studies have shown no effect of statins on cancer incidence[633,634]. Some other large studies show a benefit of statins on lung cancer incidence, although smoking habits, which are often not well documented in these studies, may confound these results[635].

In prostate cancer, the majority of studies that have investigated the influence of statins on prostate cancer incidence find no association, especially when correcting for the rate of prostate-specific antigen (PSA) screening[636–638]. Importantly, one confounding factor for these studies is the "healthy user bias," in which chronic medication users are more likely to utilize other preventative medical resources, such as cancer screening exams (e.g. colonoscopy, mammogram, and prostate-specific antigen) and vaccinations[639].

In breast cancer, no association between statin usage and increased cancer risk has been reported in the literature[532,640]. Meta-analyses conducted by Undela et al[534] and Bonovas et al[535] show no significantly different incidence of invasive breast cancer in statin users. Other meta-

analyses that investigate the incidence of a multitude of cancers among statin users also show no difference in breast cancer incidence with statin therapy[626,628,641,642]. Similarly, in the Nurses' Health Study, no differences in invasive breast cancer incidence were observed between statin never users, former users, or current users[536]. Finally, the Women's Health Initiative demonstrated no difference in incidence of breast cancer between statin users and non-users[643]; however, a decrease in the diagnosis of advanced stage breast cancer was seen in users of lipophilic statins[644].

In summary, statin usage appears to decrease the incidence of certain cancer types but not that of others. Several studies suggest statins reduce the risk of primary liver cancer. Some studies suggest statins reduce the risk of colorectal carcinoma, yet previous statin users carry a similar risk to current users. The majority of evidence suggests statins do not affect the incidence of colorectal, lung, or prostate carcinoma. Similarly, in breast cancer, incidence is unaffected by statin usage; however, diagnosis of later stage disease may be reduced with statins. In conclusion, statins appear to have no effect on the incidence of primary breast cancer.

2.6.1.2 Statin Influence on Cancer Recurrence

Fewer studies have investigated the influence of statins on cancer recurrence than incidence and mortality. Wu et al demonstrated a decreased recurrence of hepatitis B virus-related primary liver cancer after liver resection in patients taking statins[645]. In colorectal cancer, studies by Lash et al[646] and Ng et al[647] suggest no influence of statins on cancer recurrence in patients with stage III or stage I - III disease respectively. In lung cancer, statins have been associated with a decreased local recurrence in non-small cell lung cancer (NSCLC)[648] and an increased progression free survival (PFS) in NSCLC patients receiving EGFR tyrosine kinase inhibitor therapy[649]. A randomized phase III clinical trial that investigated adding pravastatin to standard chemotherapy in patients with small cell lung cancer (SCLC) showed no influence of the statin on PFS, but this study used

a hydrophilic statin and had a relatively small sample size (n = 846)[650]. The majority of the studies that probe the influence of statins on cancer recurrence have been conducted in the context of prostate or breast cancer.

In prostate cancer, the influence of statin usage on disease recurrence is dependent on the treatment modality. For patients who receive primary radical prostatectomy, no influence of statin usage on cancer recurrence is observed[651–653]. However, in patients receiving radiotherapy, either by external device or internal delivery (brachytherapy), a benefit of statin usage on prostate cancer recurrence is seen[654]. Gutt et al demonstrate statin use in men treated with radiation therapy resulted in a significant increase in recurrence free survival (RFS) and biochemical free survival (BFS)[655]. Soto et al show an improvement in 5-year PFS rate in statin users vs non-users in patients receiving radiotherapy for localized prostate cancer[656]. Oh et al show statin users receiving permanent iodine-125 brachytherapy results in a significant increase in BFS[657]. In head to head comparisons, both Tan et al[651] and Park et al[652] demonstrated a benefit of statins on BFS and RFS respectively, in prostate cancer patients receiving radiotherapy but not those treated primarily with radical prostatectomy. In summary, statin usage reduces prostate cancer recurrence in patients treated with radiotherapy but not those treated with radical prostatectomy. Since the incidences of radical prostatectomy and curative radiotherapy in locally advanced prostate cancer are 14% and 52% respectively, the statin recurrence benefit will apply to the majority of prostate cancer patients[658].

In breast cancer, the majority of clinical evidence supports a protective effect of statins on reducing breast cancer recurrence[532]. The first strong evidence, presented by Kwan et al, demonstrated a significant reduction in breast cancer recurrence in early stage patients using lipophilic statins[659]. A study conducted by Chae et al demonstrated a reduction in recurrence of

stage II and III breast cancers in patients taking statins, approximately 90% of which were lipophilic statins[660]. The benefits of statins on reducing breast cancer recurrence appears to be the strongest in younger patients, suggesting a longitudinal influence of statin therapy[661]. Boudreau et al showed that statin users have a trending lower risk of recurrence in the contralateral breast, although the study was underpowered[662]. A large meta-analysis conducted by Manthravadi and colleagues demonstrated a 36% reduction in the risk of breast cancer recurrence in patients taking statins[663]. Importantly, when the authors separated studies using lipophilic and those using hydrophilic statins, they found a significant reduction in breast cancer recurrence in patients taking the former but not the latter[663]. The most convincing evidence in the literature was a population study of all Stage I – III breast cancer patients in Denmark conducted by Ahern and colleagues[664]. They demonstrated a 10% reduction in breast cancer recurrence among women who were prescribed a lipophilic statin (most commonly simvastatin) but an identical risk in recurrence among women who were prescribed a hydrophilic statin[664]. These data in aggregate suggest lipophilic statins, and not hydrophilic statins, reduce the risk of breast cancer recurrence.

In summary, statins reduce the risk of recurrence of most cancer types. While statins can reduce the recurrence of primary liver and lung cancer, they do not appear to affect the recurrence of colon cancer. In prostate cancer, statins reduce the risk of recurrence in patients treated with radiotherapy but not those treated by primary radical prostatectomy. In breast cancer, lipophilic but not hydrophilic statins significantly reduce the risk of recurrence, irrespective of breast cancer treatment modalities. Since approximately 20% of statin users take a hydrophilic statin[604], they would not be expected to benefit from a reduction in cancer recurrence.

2.6.1.3 Statin Influence on Cancer Mortality

Similar to cancer recurrence, the majority of published data suggest that statins have a mortality benefit in a multitude of cancer types. In primary liver cancer, pravastatin and any statin usage reduces cancer-specific mortality[665–667]. Similarly, in colorectal cancer, statin usage reduces cancer specific mortality, in particular when used either prior to diagnosis or prior to recurrence[646,668,669]. In lung cancer, retrospective studies have shown that statins reduce cancer-specific mortality[649,670,671]. LUNGSTAR, a phase III randomized clinical trial, found no influence of pravastatin on the survival of patients with SCLC[650]. This result is partly expected, as pravastatin is hydrophilic, meaning its uptake by tumor cells is limited.

In prostate cancer, statin usage significantly reduces cancer-specific and all-cause mortality. A meta-analysis by Tan et al demonstrated a 32% lower risk of prostate cancer-specific mortality (PCSM) in statin users[651]. A similar meta-analysis by Meng et al demonstrated a 36% decrease in PCSM in pre-diagnosis statin users[672]. Interestingly, a smaller (n=760) matched case-control study demonstrated a 50% reduction in PCSM by both lipophilic and hydrophilic statins[673]. This equivalence of lipophilic and hydrophilic statins could be due to OATP2B1 expression on prostate cancer cells, which has been previously shown to mediate the uptake of both hydrophilic statins and testosterone precursors[612,613]. Finally, in a retrospective study of all men in Denmark with prostate cancer, post-diagnosis statin use was found to reduce PCSM by 17%, suggesting statins exert longitudinal survival benefits in this patient population[674].

In breast cancer, the majority of studies suggest statin usage significantly reduces breast cancer-specific mortality (BCSM). A meta-analysis by Liu et al demonstrated a 43% reduction in BCSM in women with breast cancer, which was confined to lipophilic statins[675]. A nationwide cohort study in Finland by Murtola and colleagues demonstrated a 46% and 54% reduction in

BCSM for pre- and post-diagnosis statin users respectively[676]. A nationwide cohort study in the UK by Cardwell et al demonstrated a 16% reduction in BCSM, which was higher in patients taking simvastatin, a lipophilic statin[677]. Finally, a nationwide cohort study in Scotland by McMenamin et al demonstrated a 15% reduction in BCSM in pre-diagnosis statin users[533].

In summary, statin usage reduces cancer-specific mortality in primary liver, colorectal, lung, prostate, and breast cancer. Importantly, this survival benefit is preferentially observed in lipophilic statin users, except for prostate cancer in which hydrophilic statins may influence steroid hormone synthesis by reducing testosterone precursor uptake[612,613]. Two additional studies provide convincing data for an overall benefit of statins on all site cancer-specific mortality (ASCSM). First, a meta-analysis by Zhong et al showed a 31% and 23% reduction in ASCSM in pre- and post-diagnosis statin users respectively[678]. Second, a nationwide cohort study in Denmark demonstrated a 13-17% reduction in ASCSM in pre-diagnosis statin users as compared to never-users. These data suggest both pre- and post-diagnosis statin usage can reduce cancer-specific mortality.

2.6.1.4 Conclusions

In conclusion, many clinical studies have demonstrated that statins can influence carcinogenesis and cancer progression. While statins do not affect the incidence of most cancers, they do appear to reduce the risk of developing primary liver cancer, likely due to the inherent selectivity of statins for hepatocytes. In contrast, statins exert significant benefits on recurrence and survival in many cancer types, including primary liver, lung, colorectal, prostate, and breast. Importantly, statin recurrence and mortality benefits are most strongly seen with lipophilic statins. These clinical studies motivate further investigation of statins in basic research studies and targeted clinical trials to evaluate potential additive or synergistic effects of statins when used with standard therapies.

2.6.2 Basic Research Studies

The clinical studies discussed above have been complemented by basic research studies that focus on the mechanisms by which statins exert anti-tumor effects. There are four main categories of effects statins exert on tumor cells: 1) growth suppression, 2) apoptosis induction, 3) anti-invasive and anti-metastatic, and 4) anti-angiogenic.

2.6.2.1 Statin Suppression of Cancer Cell Growth

Previous *in vitro* studies using leukemia[679], hepatocellular[680], bladder[681], esophageal[682], prostate[683], lung[684], cervical[685], colorectal[686], and breast carcinoma[539] cells have demonstrated that statins can halt proliferation by inducing a G0/G1 or G2/M arrest in tumor cells. There are many pathways that have been described that contribute to this anti-proliferative effect. First, simvastatin has been shown to induce a G1 cell cycle arrest through reduction of CDK4/6 and Cyclin D1, mediated in part by increases in PPARγ[681] and glycogen synthase kinase 3β (GSK3β)[686]. Additionally, BRCA1 overexpression in breast cancer cells has been shown to sensitize cells to lovastatin treatment through regulation of CDK4/Cyclin D1[687]. Second, simvastatin, fluvastatin, and lovastatin have been shown to block the CDK2/Cyclin E mediated G1/S transition, either by direct reduction of CDK2 or Cyclin E, or by increasing expression of the CDK2 inhibitors p21 and p27[540,683,688]. Statin-mediated increases in p21 and p27 have been linked to inhibition of signal transducer and activator of transcription 3 (STAT3) and activation of AMP-activated protein kinase (AMPK) in hepatocellular carcinoma cells[680]. Third, statins block DNA-binding activity of NF-κB and activator protein-1 (AP-1), which results in decreased transcription of genes that regulate cell proliferation[537,590]. For example, statin-induced suppression of NF-κB upregulates PTEN, which results in a decrease of Akt-mediated growth signaling[538,689]. Fourth, statins inhibit activation of

activator protein-1. Fifth, statins have been shown to inhibit proliferation of breast cancer cells by suppressing FPP and GGPP modification and activation of Ras, Rac, and Rho small GTPases[690,691]. Rho GTPases normally play an important role in p27 degradation, meaning inactivity results in an accumulation of p27 and cell cycle arrest[692,693]. Additionally, Rho GTPases phosphorylate and activate YAP and TAZ, transcriptional co-activators in the Hippo pathway, which mediate transcription of genes involved with proliferation[694]. Statin-mediated decreases in Rho GTPase activity thus result in decreased YAP/TAZ activity and decreased cell proliferation[695]. Finally, statins have been shown to inhibit DNA methyltransferases, altering gene transcription and reducing cancer cell proliferation[696,697].

In summary, statins act to suppress cancer proliferation by inducing cell cycle arrest, blocking proliferation signals, and influencing gene transcription. Statins cause G1 cell cycle arrest through regulation of CDK4/Cyclin D1 and CDK2/Cyclin E, in part by causing accumulation of the CDK2 inhibitors p21 and p27. Statins block proliferation signals from Ras, Rac, and Rho GTPases, as well as upregulate PTEN, which results in decreased PI3K pathway activity. Finally, statins can modulate gene transcription by limiting NF-κB and AP-1 DNA binding and by reducing DNA methylation.

2.6.2.2 Statin Induction of Cancer Cell Apoptosis

In addition to suppressing growth of tumor cells, there are some reports in the literature that statins can induce cancer cell apoptosis. There are several pathways that have been described that contribute to induction of apoptosis. First, statins have been shown to decrease the protein levels of anti-apoptotic proteins such as Bcl-2 and Bcl-xL[698,699]. For example, simvastatin can reduce Bcl-2 and Bcl-xL expression in PC-3 cells and reduce Bcl-2 expression in MCF-7 cells. Second, statins have also been shown to upregulate the activation of pro-apoptotic molecules such as Bax,

Bad, and Caspases 3, 8, and 9[541,693,698–701]. For example, lovastatin and simvastatin can induce cleavage and activation of caspases 3, 8, and 9 in prostate cancer cells[693], and simvastatin and fluvastatin can increase capsase-3 activity in breast cancer cells[702,703]. Additionally, statins have been shown to decrease phosphorylation and degradation of Bim in breast cancer cells[704], which promotes apoptosis. Third, statins can induce reactive oxygen species (ROS) generation, resulting in an increase in p38 MAPK and activation of apoptotic pathways[539,705]. Similarly, iNOS-mediated generation of nitric oxide was shown to contribute to simvastatin and fluvastatin induced apoptosis in breast cancer cells.[703] Fourth, statins can activate JNK-mediated apoptotic pathways that are independent of p53 activation[542,706,707]. Similarly, simvastatin has been shown to induce pro-apoptotic Erk activity in colon cancer cells[708]. Finally, statins can induce calcium-dependent apoptosis through increased mitochondrial uptake of calcium through L-type calcium channels, resulting in the release of cytochrome c[709]. It is suggested that these pro-apoptotic effects are mainly mediated by a reduction in GGPP rather than FPP modified proteins[590].

However, other studies demonstrate no influence of statins on apoptosis. For example, simvastatin was shown to decrease proliferation of bladder cancer cells without inducing apoptosis[681]. Moreover, simvastatin at physiologically relevant concentrations was shown to protect osteosarcoma cells from oxidative stress induced apoptosis by upregulation of Bcl-2[710]. While atorvastatin treatment could reduce Bcl-2 expression in MDA-MB-231 breast cancer cells, two weeks of neoadjuvant atorvastatin at 80mg/kg in human breast cancer patients was not found to alter Bax or Bcl-2 expression[706]. Moreover, other studies report minimal effects of statins on inducing cancer apoptosis[711] or a significant influence on apoptosis at concentrations much higher than currently used clinically[590,712].

In summary, statins have been shown to induce apoptosis in many different cancer cell lines, including prostate, breast, and colorectal cancer. The proposed mechanisms of apoptosis induction involve an upregulation in pro-apoptotic pathway members (e.g. Bax, Bad, Bim), a downregulation in anti-apoptotic pathway members (e.g. Bcl-2 and Bcl-xL), increased caspase activation, increased ROS generation, and increased calcium-dependent apoptosis. However, these effects are typically seen with statin doses much higher than used clinically. Moreover, other studies demonstrate no influence of statins on apoptosis in both *in vitro* and clinical models of cancer. Thus, the precise mechanism and magnitude of statin-induced apoptosis of cancer cells remains unclear.

2.6.2.3 Statin Suppression of Angiogenesis

Statins exert biphasic effects on angiogenesis. While statins exert pro-angiogenic effects at low (nanomolar) concentrations, they exert anti-angiogenic effects at higher (micromolar) concentrations[713]. At low concentrations, statins can induce mobilization and differentiation of endothelial progenitor cells (EPCs) through stimulation of the PI3K-Akt pathway[714]. Similarly, statin treatment can induce proliferation in EPCs through upregulation of cyclin genes and downregulation of the CDK2 inhibitor p27[715]. Moreover, low statin concentrations can enhance endothelial cell migration and tube formation in a PI3K-Akt and endothelial nitric oxide synthase (eNOS) dependent manner[716]. The PI3K-Akt pathways seem to be required for statin-mediated angiogenesis, as blocking PI3K eliminates pro-angiogenic effects of statins[717]. Additionally, statin-mediated activation of eNOS is dependent on the ability of heat shock protein 90 (Hsp90) to recruit active Akt to eNOS, which results in nitric oxide stimulated angiogenesis[718,719].

In contrast, at higher concentrations, statins negatively impact angiogenesis through multiple mechanisms. First, statins have been shown to reduce membrane localization of RhoA by

blocking its geranylgeranylation, which reduced the tube forming ability of human endothelial cells *in vitro* and was reversed with GGPP supplementation[720]. This inhibition of RhoA subsequently suppresses signaling through pathways that require RhoA activity, such as VEGFR, FAK, and Akt[720]. Second, statins decrease the abundance of caveolin-1 in endothelial cells, which reduces VEGFR2-mediated angiogenic signaling in a cholesterol-independent manner[721,722]. Third, high doses of statins can induce JNK-, Bax/Bcl-2, and caspase-mediated apoptosis of endothelial cells[713,716,723]. Fourth, endothelial cells treated with relatively higher doses of statins show a decrease in hypoxia-stimulated secretion of VEGF and expression of VEGFR2[713]. Finally, simvastatin has been shown to upregulate vascular epithelial cadherin (VE-cadherin), which limited endothelial cell proliferation, migration, and tube forming ability.

In the context of cancer, statins can reduce tumor angiogenesis through several different mechanisms. First, statins can directly affect the endothelial cells to reduce tumor angiogenesis. For example, simvastatin and lovastatin treatment were both shown to decrease tumor vascularization in mice at high statin doses[713,724]. Second, statins can act indirectly on the endothelium by reducing pro-angiogenic or increasing anti-angiogenic protein secretion by tumor cells[725]. For example, lovastatin was shown to suppress VEGF secretion by tumor cells *in vitro*[724]. Similarly, atorvastatin has been shown to reduce circulating VEGF concentrations in human patients with coronary artery disease[726]. Moreover, simvastatin has been shown to decrease secretion of angiopoietin-2 (Ang-2) by colorectal carcinoma cell lines[727]. Ang-2 has been previously shown to promote angiogenesis in tumors, in part through binding to cellular integrins[728–730]. Finally, simvastatin and atorvastatin can reduce matrix metalloproteinase-9 (MMP-9) expression in endothelial cells, which reduces their invasive capability[731]. Moreover, MMP-9 has been shown to be important for releasing the VEGF that is sequestered in breast cancer

ECM[732]. Thus, a reduction in MMP-9 both impacts the stimulation for and competency of endothelial cells to initiate angiogenesis.

In summary, statins exert concentration-dependent effects on endothelial cells and angiogenesis. At low concentrations, statins exert pro-angiogenic effects through stimulation of PI3K/Akt and eNOS. At high concentrations, statins exert anti-angiogenic effects through suppression of RhoA activity, which mediates several critical signaling pathways, such as VEGFR, FAK, and Akt. Moreover, high statin concentrations can induce apoptosis of endothelial cells, reduce secretion of pro-angiogenic proteins such as VEGF, and reduce availability of matrix-bound VEGF through reducing MMP-9 activity. Moreover, interactions between the tumor cells and tumor endothelium play an important role in the effects statins exert on tumor angiogenesis. Namely, statins directly decrease cancer cell secretion of pro-angiogenic factors, such as VEGF and Ang-2, which result in decreased tumor angiogenesis. Importantly, the concentration of statins that demonstrate anti-tumor effects coincide with those that exert anti-angiogenic effects. Consequently, the doses of statins that reduce cancer cell growth would negatively impact tumor angiogenesis.

2.6.2.4 Statin Suppression of Cancer Cell Invasion and Metastasis

Many *in vitro* studies have suggested statins reduce the invasiveness and metastatic potential of cancer cells through multiple different mechanisms. First, statin treatment destabilizes the cytoskeletal structure of tumor cells in a RhoA/RhoC dependent manner. Upon statin treatment, Rho delocalizes from the membrane, which causes breakdown of the actin cytoskeleton, loss of actin stress fibers and focal adhesion sites, and cell rounding[733–736]. Moreover, statin treatment is sufficient to block EGF-mediated RhoA membrane localization and formation of actin stress fiberswithout impacting EGF-mediated tyrosine phosphorylation of EGFR or HER2[736,737]. Second,

statins block adhesion of cancer cells to both ECM proteins by reducing integrin binding activity[738]. Similarly, statins downregulate E-selectin on tumor endothelial cells, which reduces tumor cell adhesion and invasion through an endothelial barrier[739,740]. Third, statins reduce expression and activity of the pro-migratory proteases MMP-2, MMP-9, and urokinase by inhibiting Ras and Rho activity[733,741,742]. Fourth, statin treatment can downregulate the cancer stem cell marker CD44 in breast cancer cells, which reduces cell migration and invasion[743]. Finally, statins can reduce expression of the transferrin receptor in breast cancer cells, which causes iron starvation and a reduction in tumor invasiveness[691,744].

Statins also impact tumor invasion and metastasis in *ex vivo* and *in vivo* models. Statins were shown to reduce prostate cancer migration towards human bone marrow stroma without impacting ECM adhesion[745]. Also, lovastatin, atorvastatin, and simvastatin have been shown to reduce metastatic seeding of melanoma and breast cancer cells in the lung and bone[734,735,743]. Importantly, these and most other studies that investigate the influence of statins on cancer metastasis inoculate tumor cells into the heart or tail vein of mice. Direct intravascular introduction of tumor cells precludes formation of a primary tumor, which is required to examine the metastatic potential of cells. As a measure of true metastatic potential, Mehta et al and Juneja et al were able to demonstrate that lovastatin could decrease metastasis of colon cancer cells that were inoculated into the spleens of mice[739,746]. Moreover, in breast cancer patients receiving 40mg simvastatin per day for 4-6 weeks before mastectomy, statin treatment was able to decrease post-explant tumor migration[747]. Moreover, simvastatin treatment reduced the activity of Rho associated protein kinase (ROCK) and expression of RhoC, chemokine receptor type 4 (CXCR4), and CD44[747].

In summary, statins can reduce the invasiveness and may reduce the metastasis of cancer cells. While the effects of the influence of statins on tumor cell migration and adhesion are well

studied, their effects on tumor metastasis remain unclear. Statins reduce tumor cell invasiveness mainly through suppression of RhoA and RhoC activity, which disorganizes the actin cytoskeleton and reduces migratory ability. Similarly, statins reduce the adhesion of tumor cells to ECM proteins and endothelial cells, in part through a reduction of integrin binding activity. Moreover, statins reduce activity of proteases involved with migration, such as MMP-2 and MMP-9.

Most existing data measure the influence of statins on tumor metastasis in mice by injecting tumor cells into the tail vein and examining lung tumor burden. Since direct intravascular inoculation of tumor cells does not allow for formation of a primary tumor, critical steps of the metastatic cascade, such as invasion and intravasation, are ignored. In studies that employ experimental models that establish a primary tumor prior to gauging metastasis, scant data suggest statins may reduce metastasis. However, the specific effects of statin treatment on tumor metastasis are not very well studied, especially in the context of breast cancer.

2.6.2.5 Conclusions

In conclusion, many basic research studies have shown that statins can reduce tumor cell growth and proliferation by inducing a cell cycle arrest. Additionally, some, but not all studies, suggest that statins can induce apoptosis in cancer cells. In addition to affecting tumor cell growth and apoptosis, statins can negatively impact the tumor vasculature through suppression of angiogenesis. While statins exhibit a biphasic effect on angiogenesis, their anti-angiogenic effects are observed at similar doses to those used in studies that demonstrate anti-tumor effects. While statins have been shown to reduce cancer cell migration and invasion *in vitro*, few studies have demonstrated a reduction in metastasis with statin treatment *in vivo*. These previous studies motivate the investigation of statins in the context of breast cancer metastasis, in particular

dormancy and emergence, which is the main cause of morbidity and mortality in this patient population.

Proper investigation of breast cancer metastatic emergence requires an appropriate model system. The liver is one of the most common metastatic sites for breast cancer and metabolizes commonly used breast cancer treatments as well as many of the statin drugs. Previous work has shown that breast cancer co-culture with primary hepatocytes is sufficient to induce MErT in the cancer cells, mimicking the clinical scenario of metastatic seeding[45,748]. Moreover, sophisticated *ex vivo* perfusion culture systems have shown these epithelial, dormant tumor cells can be coerced to outgrow as mesenchymal metastases when exposed to an inflammatory stimulus[47,749–751]. To illustrate the suitability of the liver as a model organ for breast cancer metastasis, the physiology and pathology of liver metastasis will be discussed in the next section. Moreover, technical considerations for cell sourcing and *in vitro* and *ex vivo* culture methods will be presented.

3.0 THE LIVER AS A MODEL ORGAN FOR METASTASIS AND DRUG THERAPY

3.1 PREFACE

The majority of this chapter is previously published as Beckwitt et al[1]. The purpose of this chapter is to introduce the reader to the physiology and culture systems available for replicating human liver *ex vivo*. This holds particular importance for this thesis, since most pertinent findings discuss breast cancer metastasis to the liver.

3.2 INTRODUCTION

The liver plays critical roles in both homeostasis and pathology. It is the major site of drug metabolism in the body and, as such, a common target for drug-induced toxicity and is susceptible to a wide range of diseases. In contrast to other solid organs, the liver possesses the unique ability to regenerate. Additionally, and critical for this project, the liver is the most common site for metastasis. The physiological importance and plasticity of this organ make it a crucial system of study to better understand human physiology, disease, and response to exogenous compounds.

The purpose of this chapter is to inform the reader of the significance and available methods for replicating human liver physiology and pathology ex *vivo*, in particular modeling breast cancer metastasis to the liver. First, the physiologic roles of the liver and its cellular constituents will be discussed. Second, we will discuss the need for developing an ex *vivo* liver system. Third, the advantages and disadvantages of different cell sources used to populate the system will be

mentioned. Fourth, the benefits of currently employed ex *vivo* liver culture systems (both commercially available and used in research laboratories) will be discussed. Finally, future directions to advance these systems, including complexing liver culture systems with other organ-based culture systems ("organ chips"), will be proposed.

3.3 PHYSIOLOGY

The liver is a complex organ that is integral to a multitude of whole body functions and is extensively reviewed in standard medical textbooks. For the purposes of this review, a few salient aspects are emphasized. The liver is organized into lobules, functional units of the liver, which are subsequently organized into larger lobes. Lobules consist of multiple hepatic sinusoids, whose structure is illustrated in Figure 7. The liver consists of both parenchymal and non-parenchymal cells, both of which will be discussed herein.

The parenchymal cells, mainly hepatocytes, comprise 60% of the cell population and are responsible for the liver's metabolic activity (including the cytochrome P450 (CYP450) isoforms), the production and release of acute phase proteins, production of plasma proteins (such as albumin and clotting factors), and the integration of various components of glucose, lipid, nitrogen, and oxidative metabolism[752,753]. These hepatocytes mature postnatally, causing different CYP450 isoforms and assemblies thereof to be expressed in fetal, neonatal and adult hepatocytes[754]. For example, CYP3A7 is very highly expressed in fetal hepatocytes, whereas this isoform is replaced by CYP3A4 expression in adult hepatocytes. Moreover, pediatric hepatocytes typically exhibit increased clearance of CYP450-metabolized drugs as compared to adult hepatocytes[755], despite no

differences in intrinsic CYP450 expression between the two cell sources[756]. Thus, hepatocyte functioning is key to any *ex vivo* system that replicates the *in vivo* liver.

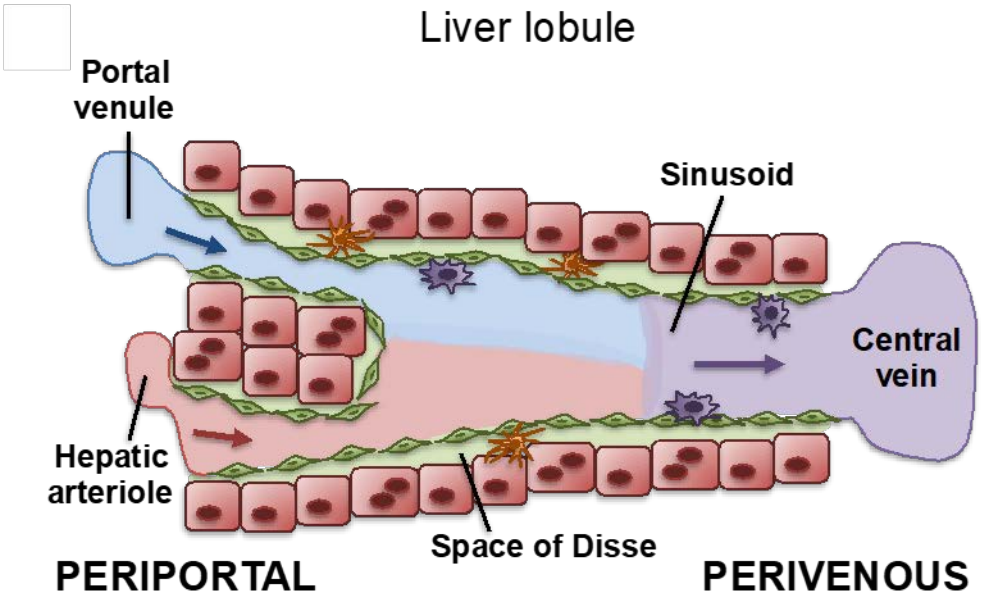


Figure 9. The architecture of the hepatic sinusoid

Blood enters the periportal region of the liver through a hepatic arteriole and portal venule and traverses the hepatic sinusoid to the central vein, whereby it is drained into the larger hepatic veins. The sinusoidal endothelial cells mediate blood flow and are fenestrated to allow diffusion of nutrients, signaling factors, and drug compounds. The hepatocytes compose the parenchyma of the liver and sit deep to the endothelium. The hepatic stellate cells reside in the Space of Disse, a zone between the endothelium and hepatocytes. Finally, the Kupffer cells line the inside of the sinusoid and mediate antigen sensing and intercellular communication.

Oxygen tension plays an important role in regulating liver zonation and functionality, with the liver uniquely receiving both arterial (hepatic artery, ~25%) and venous (portal circulation, ~75%) blood. The partial pressure of oxygen drops as one progresses across the liver sinusoid, the functional unit of the liver, from periportal to perivenous hepatocytes[757]. This oxygen differential

regulates the response of the liver to metabolic and toxic stimuli by facilitating differential metabolism, termed "liver zonation." For example, the relatively hypoxic perivenous hepatocytes are responsible for the majority of substrate metabolism through the CYP450 system whereas the relatively oxygen-rich periportal hepatocytes boast mainly oxidative metabolic functions[757]. Liver zonation is also observed in *in vitro* cultures[758,759]. Thus, the organization of the parenchyma motivates careful engineering to replicate hepatic function and toxicity and capture the full panoply seen *in vivo*.

Blood flow carried by the fenestrated endothelium of the sinusoids is imperative for liver functions. Hepatic blood flow dictates the metabolic zonation of the liver[757] and alterations in flow can reduce liver function and drug clearance[760]. Blood flow carries oxygen, nutrients, and chemicals that are distributed to the liver tissue. In the absence of adequate blood flow, these factors are consumed by the proximate cells and do not reach more distant populations. Reciprocally, juxtavascular cells can contribute factors to the circulation. Locally, the potency of hepatocyte-secreted factors is a function of the blood-to-cell volume ratio, which is approximately 20.5 mL blood per mL tissue for healthy adult livers[761]. Assuming a hepatocyte volume of 3.4 pL[762], this corresponds to 14 million hepatocytes being supplied by 1 mL of blood. This consideration is particularly important for *in vitro* and *ex vivo* culture systems, as media flow is used by some to model *in vivo* blood flow (see "Engineered Culture Systems").

The non-parenchymal cells (NPC) compose the remaining 40% of the cell population and play a significant role in tissue architecture and in mediating responses of the tissue to metabolic and toxic stimuli, as well as supporting the hepatocyte function[753,763]. These cell types include liver sinusoidal endothelial cells (LSECs), Kupffer cells (KCs), hepatic stellate cells (HSCs), and pit cells (natural killer cells, NKs). Inclusion of NPCs in hepatocyte culture systems has shown

beneficial effects. For example, 3-dimensional (3D) liver tissue models show increased hepatocyte functions when NPCs are incorporated[764]. Additionally, KCs play a significant role in the response of the liver to injury through the production of cytokines and reactive oxygen species[765]. Moreover, HSCs respond to injury both by adopting a myofibroblast phenotype that remodels the liver extracellular matrix[766] and increasing the CYP450 activity of hepatocytes[767]. Finally, LSEC proliferation in response to injury has been suggested to aid the liver's potent regenerative capacity[768]. Thus, the NPCs complement the synthetic and metabolic functions of hepatocytes by contributing pro-regenerative, pro-inflammatory, and pro-fibrotic stimuli.

3.4 THE LIVER AND CANCER METASTASIS

The liver is host to many pathologies and is a common site for drug toxicity (see below). For the purpose of this dissertation, a brief but sufficient introduction to the liver as a common site of metastasis will be provided. Other disease pathologies in the liver will be covered only in the context of culture systems that have been designed. For a more comprehensive review of liver diseases, the reader is directed to more comprehensive sources[769].

The most common malignant liver lesion is a metastasis. Indeed, their prevalence is 18-40 times higher than primary tumors[770]. Moreover, 25-50% of patients with solid organ tumors will present with liver metastases at diagnosis[771]. The most commonly observed metastases in the liver are derived from colon cancers, due in a large part to their easy accessibility to the portal circulation[772]. However, almost every cancer type has the ability to metastasize to and colonize the liver, with the most common cancer types being carcinomas derived from colon, lung, pancreas, and breast. Data from a large autopsy study of 23,000 patients are shown below in Figure 8[773].

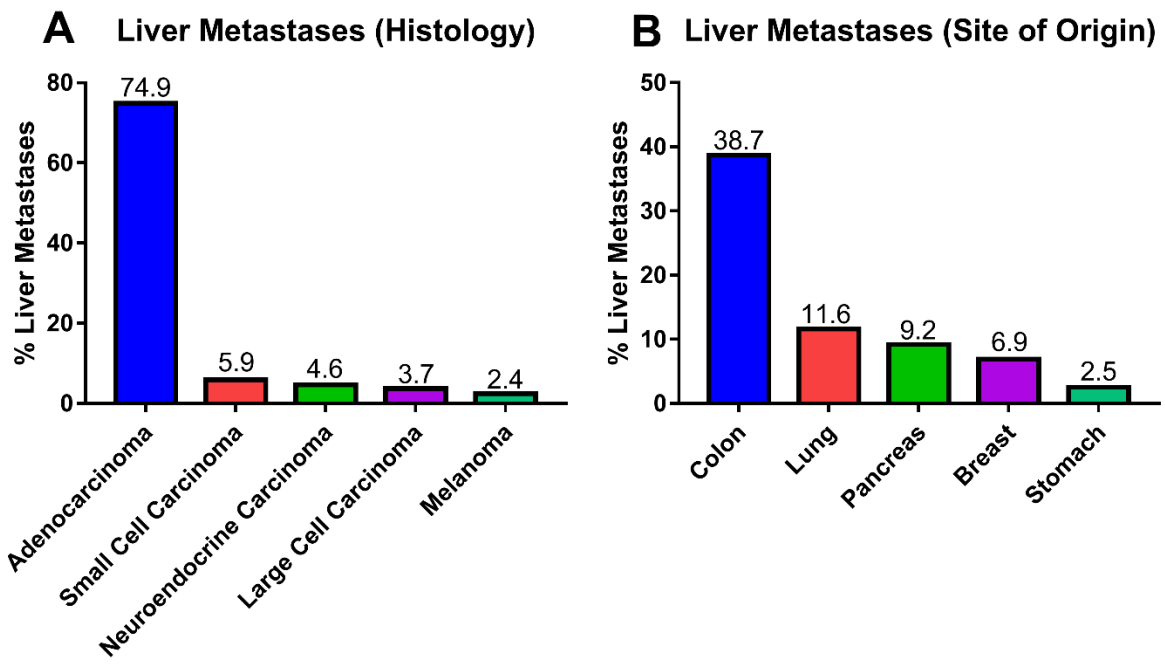


Figure 10. Liver metastases histology and site of origin

(A) Histologic classification and (B) site of origin of metastases found in livers of 23,154 patients. Graphs made from data in the supplement of de Ridder et al 2016[773].

The high prevalence and morbidity of liver metastases makes this organ the ideal system for studying metastatic colonization, outgrowth, and response to therapeutic compounds. The next sections will detail the important design parameters for generating a suitable culture system that robustly mimics the physiology of the normal liver.

3.5 MODELING THE LIVER MICROENVIRONMENT

Current preclinical models for hepatotoxicity involve human cell culture and animal models. Recently, efforts to develop *ex vivo* hepatic culture systems, "liver-on-a-chip," have been undertaken by many research groups and biotech companies due to the liver's capacity for drug metabolism, excretion, vulnerability to drug-induced damage, and as a primary organ in many diseases. Drug-induced liver injury remains a major reason for drugs being withdrawn from the market, and causes both morbidity and mortality for patients. Importantly, humans metabolize and respond to agents differently from other mammals; to the point, most all species present unique xenobiotic handling[774]. In fact, one-third of toxicities observed in humans are not predicted in any of the species commonly employed for drug safety testing[775], possibly due to their failure to model reactive metabolites generated through human-specific metabolic pathways[776]. Moreover, individual animal models have a success rate of as low as 40% in predicting hepatotoxic compounds[777], resulting in 26% of clinical trial failures being due to hepatotoxicity[778].

Current liver tissue culture systems exist on a spectrum of complexity. Historic hepatocyte culture systems involved collagen-sandwich culture or 2D Micro-Patterned Co-culture (MPCC) systems using primary rat hepatocytes and 3T3-J2 fibroblasts. Systems have progressed to include 3D static spheroid models and perfusion culture devices, which introduce nutrient and oxygen gradients and shear stress that are important for hepatocyte functions[779]. The systems discussed below offer distinct advantages and disadvantages for investigating the response of hepatic micro-tissues to different drugs and other stimuli.

3.6 CELL SOURCING

The complex physiology of the liver and need for its accurate representation in engineered systems requires careful selection of cell type(s) and their origin. Hepatic tissue is composed of hepatocytes (60% of liver cells) and a complex complement of NPCs (40% of liver cells). Integration of both cell fractions is often needed to adequately reflect pharmacokinetics, pharmacodynamics, toxicity of drugs, and liver disease progression, given the intercommunication between the different liver cell types. Four sources of hepatocytes will be discussed: primary human cells, primary animal cells, immortalized human cell lines, and pluripotent stem cells. Each of these cell sources has its advantages and disadvantages, and each will be discussed below. A qualitative summary of cell selection parameters can be seen in Table 11.

3.6.1 Primary Human Hepatocytes

Functional primary human hepatocytes (PHH) obtained through collagenase perfusion and dissociation have been the gold standard in drug discovery for 25 years due to their ability to most accurately reflect *in vivo* metabolism and toxicity[780–782]. Primary human hepatocytes have been isolated from livers with benign (processes not affecting the hepatocytes) and hepatocyte-specific pathologies. Some examples include end stage primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC), alcoholic liver disease (ALD), and resections for colorectal metastases or benign growths[783]. Several groups have found patient-specific factors to play a role in the yield of hepatocytes during isolation or in their functionality. While patient gender and type of disease had no effect on hepatocyte yield or functionality[784,785], cholestasis (signified by elevated gamma glutamyl transferase level – GGT)[784,786], severe steatosis[784,787], and older age[784,785,788,789] negatively

influenced isolation outcomes. Although some patients have been exposed to chemotherapy, hepatocyte function and viability are not altered[790]. While an ideal source, limited availability of cells and reliance on patient surgeries are major reasons why primary hepatocytes are not more commonly used. Moreover, many donors are pediatric patients, whose hepatocytes may have metabolic dysfunction or exhibit differential clearance when compared to adult human hepatocytes (see "Physiology").

Table 15. Hepatocyte sources

Hepatocyte Source	Animal	HepG2	HepaRG	Primary Human	Cryopreserved Human	iPSC-derived
Viability	Medium to High*	High	High	Medium to High*	Medium	High
Attachment to ECM	High	High	High	High	Medium	High
CYP450 Activity	Medium	Low	Medium	High	High	Low to Medium
Longevity in Culture	Medium	High	High	Medium	Low to Medium	Low
[1]OATP/NTCP	High	Low	Medium	High	Medium	Low to Medium
Cost	Medium	Low	Low	High	High	High
Availability	Medium	High	High	Low	Medium	Medium
References	791–794	795–797	796–801	779–781	790,802–804	805–807

A summary of properties from different hepatocyte sources: animal, human cell line, primary human, and iPSC-derived. *Dependent on efficiency and time of isolation procedure. [1]OATP (organic anion transporting polypeptide), NTCP (sodium taurocholate co-transporting polypeptide).

Advances in cryopreservation have allowed for easier distribution and commercialization of primary human hepatocytes. Previous work has demonstrated cryopreserved primary human hepatocytes to still exhibit 94% of the clearance of model drugs (e.g. diclofenac) after 1 year in liquid nitrogen, as compared to freshly isolated human hepatocytes[802]. With proper cryopreservation, viabilities >90% can be achieved[808]. Additionally, minimal changes in metabolic

functions have been demonstrated even after 14 years in liquid nitrogen[809]. While cryopreserved primary human hepatocytes exhibit high viability and comparable CYP450 activity to fresh cells, they exhibit suboptimal attachment to extracellular matrix molecules, due to downregulation and degradation of the adhesion molecules β1-integrin and E-cadherin respectively during cryopreservation[803]. Additionally, decreased expression of uptake transporters such as OATP (organic anion-transporting polypeptide) and NTCP (sodium-taurocholate co-transporting polypeptide) family members results in under-prediction of drug clearance rates[810]. Finally, high viability methods often involve centrifugation to remove dead and damaged hepatocytes, which also reduces the cell yield.

Cryopreserved primary human hepatocytes have been used in numerous studies to investigate CYP450 induction[804], drug clearance[811], and hepatobiliary transport[812]. In contrast to human hepatic cell lines or animal primary cells, cryopreserved primary human hepatocytes can probe the influence of genetic background on hepatocyte metabolism or drug clearance. For example, a collection of cryopreserved hepatocytes from 64 donors has been used to investigate gender-specific activities of CYP450 isoforms *in vitro*, which corroborated with *in vivo* findings, namely a higher expression of CYP3A4, the most dominant human isoform, in females[813]. Thus, while human cell lines and animal hepatocytes are more accessible, primary human hepatocytes will likely continue to play an important role in drug development due to their ability to most effectively reflect *in vivo* metabolism, clearance, and response to toxins by patients.

3.6.2 Animal-Derived Hepatocytes

Animal-based testing has formed the foundation for translating *in vitro* studies to clinical trials. Before moving to humans, the Federal Drug Administration (FDA) requires pharmacokinetic,

toxicity, and efficacy studies in at least one rodent (mouse, rat) and one non-rodent (dog, rabbit) species. Such studies are designed to examine integrated organ responses and are able to suggest, at least initially, the influence of genetic diversity on drug responses[791]. Additionally, these species are readily commercially available, thus serving as sources of fresh primary cells. However, animal studies frequently do not elicit the pharmacokinetic behavior seen in humans due to species differences in drug metabolism and clearance, in particular differential CYP450 metabolic activity[792,793]. Differences in CYP450 induction by model drugs between human and animal (rat, dog) are well established[814]. Improvements in predicting human-specific metabolism and clearance from animal studies have been made through retrospective analyses[794]. Yet, sourcing cells from animals may be cost prohibitive, ethically controversial, and futile if interspecies differences prevent human-relevant data from being collected[815]. Moreover, since animal hepatocytes express different CYP450 isoforms than humans, extrapolating of gender-specific differences in drug metabolism is challenging[816].

3.6.3 Human Hepatic Cell Lines

Human hepatic cell lines, either cancer-derived or immortalized hepatocytes, can be effectively propagated in culture over multiple passages. Some of the most commonly used hepatic cell lines include HepG2, Huh7, Hep3B, and SK-Hep-1, all derived from hepatocellular carcinoma (HCC), and HepaRG, an HCC cell line that constitutes a mix of both hepatocytes and biliary-like cells[795,796].

One issue with using human hepatic cell lines is that they exhibit lower and variable CYP450 expression than primary human hepatocytes[796]. For example, CYP3A4 is not expressed in HepG2 or Hep3B cells whereas CYP2D6 is expressed at less than 5% the level of primary

human hepatocytes. Studies in HepG2 cells demonstrated that CYP450 expression may also vary according to culture duration and passage number, some isoforms varying up to 200-fold across the first 10 passages[817]. Some cell lines hold more promising results. For example, induction of CYP450 enzymes and the drug transporter MDR1 was seen in a novel hepatic cell line, Fa2N-4, in response to rifampin treatment[797].

Another issue with human hepatic cell lines is that they exhibit reduced expression of both sinusoidal and canalicular transporters[798]. These transporters function to shuttle drug compounds from the blood to the bile canaliculi. While hydrophobic compounds can diffuse across the hepatocyte plasma membrane, hydrophilic compounds require active transport. Reduced transport activity and decreased CYP450-catalyzed metabolism may result in inaccurate pharmacokinetic and toxicity predictions.

The HepaRG cell line is a promising substitute for primary human hepatocytes. When seeded at a low density, HepaRG cells are capable of proliferating and differentiating to confluency to form colonies of hepatocytes surrounded by biliary epithelial cells that exhibit CYP450 expression levels comparable to primary human hepatocytes[818]. HepaRG cells have also been found to functionally express both sinusoidal and canalicular transporters[799,800]. Additionally, HepaRG cells can identify drugs likely to induce liver injury, albeit using doses up to 100-fold higher than would be seen clinically[801]. Moreover, HepaRG cells exhibit a more robust response to inflammatory stimuli (e.g. IL-6, TNFα) on liver drug metabolism and elimination than primary human hepatocytes, potentially due to genetic variation in the latter[819].

While hepatocyte cell lines are effective in predicting the metabolism and elimination of some drugs, their overall sensitivity to detect toxic compounds is lower than primary human hepatocytes (13% for HepaRG compared to 44% for PHHs)[795]. Further, being derived from a

single female donor, these cells do not account for genetic variation in drug responses that often uncover limiting toxicities in a subset of persons. This limitation is also seen with primary hepatocytes, as testing is often performed on a limited number of donors. Importantly, since human hepatic cell lines are derived from primary hepatic tumors, they may not accurately represent normal cellular responses. These weakness ultimately require the use of complementary approaches (i.e. normal primary cells)[779].

3.6.4 Induced and Embryonic Pluripotent Stem Cells (iPSC and ESC)

Stem cells are defined as cells capable of self-renewal and differentiation into mature cells of a particular tissue type[820]. Stem cells encompass both those derived from blastocysts (ESC) and also those derived from mature cells through reprogramming (iPSC) using viral vectors, Cre-lox expression cassettes, or mRNA/miRNA transfection[821]. While iPSC generation and differentiation is time consuming, it allows for an easily sharable cell population with theoretically limitless growth potential that can mimic preliminary clinical trials *in vitro*, hopefully improving the success in subsequent human studies.

Many protocols demonstrate differentiation of iPSC[822,823] or ESC[824–826] into hepatocyte-like cells. Protocols that try to replicate liver embryogenesis can achieve hepatocyte-like cell yields of up to 80%[826]. Additionally, the expression of microRNAs (mir), specifically mir-122, during the differentiation process can further improve hepatocyte fidelity[805]. However, many differentiation protocols result in hepatocytes that express immature markers (e.g. alpha-feto protein; AFP), secrete less albumin, and exhibit dramatically reduced CYP450 activity than primary human hepatocytes[806,807,827]. Indeed, hepatocyte-like cells differentiated from iPSC using multiple sources and two different protocols demonstrated a phenotype akin to fetal hepatocytes[828].

An advantage of iPSCs is the potential to study a broad spectrum of hepatocyte lines with different genetic, epigenetic backgrounds[807,828] and disease backgrounds. Additionally, iPSCs exhibit sensitivity comparable to primary human hepatocytes for detecting drugs that cause drug induced liver injury (65% vs 70%)[829]. While a promising technology, current iPSC technical limitations include epigenetic memory, genomic instability, state of maturation to adult cells and variability among cell lines. Strategies to address these weaknesses include using small molecule treatment, avoiding targeting p53 during reprograming, and use of multiple iPSCs from different genetic backgrounds, respectively[830]. Namely, platforms aimed to analyze the influence of small molecules on hepatocyte expansion and differentiation of iPSCs to mature hepatocytes have shown promising results[831]. However, a steady supply of hepatocyte-like cells derived from iPSC is often not available to many researchers, which limits consistent use to labs with experience in their differentiation. Encouragingly, some companies are starting to commercialize iPSC-derived hepatocytes.

3.6.5 Non-Parenchymal Cells (NPCs)

The NPC component of the liver is critical to recreating physiologic liver functioning and response to injurious stimuli. As such, inclusion of NPCs is desirable to replicate metabolism and signaling pathways observed *in vivo*. Addition of NPCs in hepatic culture systems has shown to alter signaling networks present in the microenvironment, enhance hepatocyte synthetic functions, prevent hepatocyte de-differentiation in culture, and enhance hepatocyte metabolic response to drug treatment[752,832,833]. Both primary and immortalized cell types are available and will be discussed below.

Primary NPCs are isolated in a similar manner to primary hepatocytes, by liver enzymatic perfusion. These cells can either be harvested alone or simultaneously with primary hepatocytes. When harvested alone, the NPCs of the liver can be enriched, at the expense of hepatocyte recovery, by using a pronase-collagenase perfusion method[834,835]. For isolating both cell components, collagenase perfusion followed by density centrifugation are used to collect and separate the hepatocytes from the NPCs, respectively. Several methods have been used to purify the NPCs, which include iodixanol or percol density gradients and magnetic activated cell sorting[836–838]. Importantly, the disease state of the human donor tissue can greatly influence the quality and quantity of cells obtained[837,839]. To avoid the need for donor tissue, others have demonstrated generation of NPCs from human iPSCs[840].

While primary NPCs are the most effective at reproducing the microenvironment seen physiologically, their main disadvantages are non-specific activation and availability. Using primary NPCs requires having surgical specimens. Cryopreserved NPCs can be purchased commercially either as a collective or for specific NPC types. Due to the high cost of primary cells, various cell lines or alternative cell types have been employed in co-cultures with hepatocytes to provide similar trophic signals as primary NPCs. The next several sections will illustrate approaches to incorporate each NPC type into hepatic culture systems.

3.6.5.1 Endothelial Cells

Liver sinusoidal endothelial cells (LSECs) are a unique endothelial cell type displaying abundant fenestrations, high endocytotic ability, and ability to provide ideal trophic support to hepatocytes. These cells are often unstable *in vitro*[841–844] and are difficult to cryopreserve[845]. As such, some groups have instead used human umbilical vein endothelial cells (HUVECs) that, in co-culture with hepatocytes, improve hepatocyte specific function, such as CYP450 activity and albumin

synthesis[842,846,847]. However, as HUVECs are a unique cell type that lack appreciable levels of key cell surface receptors CXCR3[848] and EGFR[849], other primary human endothelial cells have been employed with variable results[842,850].

Immortalized endothelial cell lines have also been used in hepatocyte co-culture. HMEC-1 (human foreskin endothelium) cells have been shown to enhance hepatocyte albumin secretion[851] and can provide inflammatory cues that affect both the hepatocytes as well as metastatic cancer cells[852]. TMNK-1 cells are an immortalized LSEC cell line that has been used in hepatocyte co-culture to investigate paracrine signaling (Kawasaki et al. 2010), influence on cancer cell phenotype[763], and promote hepatocyte-specific functions in ESC-derived hepatocytes[853]. However, immortalized LSECs phenotypically resemble activated endothelial cells implicated in pathologic vessel formation in chronic liver disease[854], and may not emulate normal physiology, in particular due to their lack of fenestrations.

3.6.5.2 Kupffer Cells

KCs are implicated in host defense and are important producers of cytokines in inflammatory responses and liver diseases. Immortalized KCs have been generated in mouse and rat[855], but not human. THP-1 monocytes have been differentiated into macrophages and used in co-culture applications with both primary hepatocytes and human hepatic cell lines. Inflammatory responses were noticed in hepatocytes as a result of macrophage-secreted products[856] and direct co-culture[857]. To demonstrate the importance of KC inclusion in hepatocyte cultures, LPS stimulation of a primary hepatocyte and KC perfusion co-culture system demonstrated no effect on hydrocortisone metabolism, similar to that seen clinically[858]. However, immortalized KCs or activated macrophage cell lines do not replicate physiologic KC behavior. KCs are typically quiescent and tolerogenic due to high basal expression of TGFβ and PD1 and expression of IL-10 upon LPS

stimulation[859]. As such, alternatives to primary KCs may promote more inflammatory-related changes than seen *in vivo*. Of benefit to labs without access to fresh primary cells, cryopreserved KC are commercially available for use.

3.6.5.3 Stellate Cells

Hepatic stellate cells (HSCs) are important cells for the storage of retinoids and other lipids and are implicated in hepatocyte proliferation after partial hepatectomy, hepatic fibrosis, and portal hypertension. Freshly isolated primary HSCs quickly activate when cultured on plastic[860] and their gene expression *in vitro* does not fully reproduce that observed *in vivo*[861]. HSC quiescence can be preserved by culturing on laminin-rich gels or in suspension on a non-adherent surface[862]. Several immortalized HSC lines exist for study, including hTERT-HSC, GREF-X, LI90, TWNT-1 and more recently, LX-1 and LX-2. However, only the LX-2 cell line can be maintained in serum-free media and transfected with relatively high efficiency (30%)[863]. Importantly, while immortalized cell lines express many of the same markers as primary HSCs, their response to stimuli and basal level of activation differ from that seen observed *in vivo*[862]. Co-culture of HSCs with hepatocytes has been used to maintain hepatocyte differentiation *in vitro* by cell contact mediated signals and transfer of lipids[864].

3.6.5.4 NPC Considerations

It is clear that including NPCs in culture systems influences hepatocyte functions and the tissue microenvironment. ECs can enhance albumin synthesis and CYP450 activity in co-culture with hepatocytes. KCs can elicit inflammatory changes in response to stimuli that resemble those seen *in vivo*. Finally, HSCs are important for storing lipids that can be utilized by the hepatocytes and preserving hepatocyte differentiation. While primary non-parenchymal cells are the gold standard,

they are difficult to obtain in sufficient numbers. NPC isolation yield is low when isolated concurrently with hepatocytes and subsequent purification steps can alter NPC function. Several immortalized cell lines can serve comparable (though not equivalent) roles for each NPC type, and also offer the benefits of being easily distributable and homogeneous.

3.7 ENGINEERED CULTURE SYSTEMS

The overarching goal of all liver culture systems is to provide either a high throughput, low cost, easy to operate, and reliable system that recapitulates a reasonable fraction of human physiology, or a medium to low throughput system that reliably recapitulates a maximum of liver structure and function (liver biomimetic). This depends on the ability of the system to generate an environment that reflects the *in vivo* phenotype of the hepatocytes and NPCs, which is highly dependent on both chemical and mechanical cues. A critical issue with routine 2D primary human hepatocyte culture systems is that hepatocyte functionality rapidly declines rapidly within days[865]. To overcome this, hepatocytes have been cultured using the collagen-sandwich method. This involves seeding a monolayer of hepatocytes on a gelled layer of rat tail collagen and overlaying another gelled collagen layer on top. This culture method allows hepatocytes to maintain synthetic function (as determined by albumin mRNA) for at least six weeks[866]. However, collagen sandwich cultures do not replicate the complex multi-cellular nature of the liver and do not incorporate fluid flow, which is important for preserving *in vivo* hepatocyte functions[867]. Several of the most recent engineering advances to improve hepatocyte functions and viability in culture will be discussed in this review. These systems are demarcated into the following categories: 2D Micro-Patterned Systems, 3D Spheroid Culture Systems, and Perfusion Culture Systems. For a comprehensive review of all

advancements made in the realm of hepatocyte culture, the reader is referred to other references[781,868,869].

3.7.1 Non-Perfusion Systems

Both static and perfusion systems exist for hepatocyte culture. Static culture systems include 2D micro-patterned systems and 3D spheroid culture systems. A summary of the properties of these systems can be found in Table 12.

3.7.1.1 2D Micro-Patterned Systems

The use of 2D micro-patterned culture systems are an improvement upon standard sandwich culture as they fine-tune tissue architecture by controlling the size, geometry, and functionality of culture chambers. One commercialized system, HepatoPacTM by Hepregen, Corp. consists of micro-patterned 2D plates seeded with either rat or human hepatocytes and 3T3-J2 stromal cells. Cultures maintain hepatocyte specific functions for 4-6 weeks[870,877,878], notably albumin and urea secretion, phase I/II metabolism, and formation of canalicular networks. Publications have also reported sensitivities of 66% and 100% for corroborating hepatotoxins when tested with one or at least two cell donors, respectively[877]. The extended hepatocyte functionality may allow for accurate predictions of *in vivo* drug transporter activity[879]. Additionally, this system can be used to model CYP450 enzyme induction[878,880]. However, this system's low throughput make it less suitable than traditional monolayer culture for toxicity screening studies[880].

Table 16. Non-perfusion hepatocyte culture systems

System	Type	Cell Type(s)	CT (d)	CYP450	Urea	Albumin	Throughput
HepatocPac™ by Hepregen, Corp.[870]	2D	PHH or PRH + 3T3	28-42	Stable	Stable	Stable	Low
PDMS Stencil[871]	2D	PRH + 3T3	7	N.D.	Stable	Stable	Low
Micropatterned 96-well Plate[779]	2D	PHH or iHep + 3T3	28	Stable	Stable	Stable	High
Spheroid Microarray Chip[872]	3D Spheroid	PRH + 3T3	14	N.D.	Stable	Stable	High
Micropatterned Fibrous Mat[847]	3D Spheroid	PRH + 3T3 + HUVEC	15	Stable	↓	Stable	Low
PEG-encapsulated Spheroid[873]	3D Spheroid	PRH + 3T3	50	Stable	N.D.	Stable	High
Human Microspheroids[874]	3D Spheroid	PHH + HNPC	35	Stable	N.D.	Stable	High
RegeneTox™ by Regenemed, Inc.[875]	3D Spheroid	PHH or PRH + HNPC or RNPC	90	Stable	Stable	Stable	Medium
GravityTRAP™ by InSphero AG[876]	3D Spheroid	HepaRG	21	Stable	↑	Stable	High

A summary of properties of different non-perfusion hepatocyte culture systems. CT (d) = Culture Time in days; PHH = Primary Human Hepatocyte; PRH = Primary Rat Hepatocyte; 3T3 = 3T3 Fibroblasts; iHep = iPSC-derived human hepatocytes; HUVEC = Human Umbilical Vein Endothelial Cells; HNPC = Human Non-Parenchymal Cells; RNPC = Rat Non-Parenchymal Cells; N.D = Not determined.

Similar systems have used micro-patterned hepatocyte islands to enhance hepatocyte functioning in 2D culture. For example, Cho and colleagues used polydimethylsiloxane (PDMS) stencils to co-culture rat hepatocytes and 3T3 fibroblasts and demonstrated enhanced hepatocyte functions when co-cultured in a layered format (hepatocytes seeded on an island of fibroblasts) as compared to a co-planar format[871]. Ware and colleagues used micro-patterned hepatocyte-murine fibroblast co-cultures to predict hepatotoxicity using both primary human hepatocytes and iPSC-derived hepatocyte-like cells[829]. However, these systems do not take advantage of the positive

influence of LSECs on hepatocyte functions, as previously shown with hepatocyte co-culture with HUVECs[881].

Since these systems involve a monolayer of cells, they are amenable to high content image analysis without confocal optics. However, these systems have not been fully characterized for co-culture with liver-specific NPCs and are patterned using rat tail collagen I rather than liver specific matrix proteins[779]. Previous studies have shown that culturing hepatocytes on liver-specific ECM leads to higher attachment efficiency and lower expression of the dedifferentiation markers vimentin and cytokeratin 18[882]. Moreover, supplementation of liver-specific ECM digests in hepatocyte cultures showed improved albumin synthesis and CYP450 activity[883,884]. Enhanced albumin secretion and cellular connectivity is also observed with less stiff culture substrates, such as heparin gels, with the least physiological morphology observed on collagen coated glass[885]. These findings suggest culture materials that mimic physiologic liver extracellular matrix promote maintenance of *in vivo* hepatocyte functions.

3.7.1.2 3D Spheroid Culture Systems

Three dimensional culture systems add an additional layer of complexity by more accurately representing the tissue architecture of a whole liver. Micro-patterning and functionalizing surfaces can facilitate the formation of 3D hepatocyte spheroids. For example, Fukuda and colleagues micro-patterned cylindrical culture wells with collagen and polyethyleneglycol, which facilitated rat hepatocyte spheroid formation. These spheroids demonstrated enhanced albumin secretion and ammonia detoxification compared to monolayer culture for up to 14 days in culture[872]. They also used a photo-crosslinkable chitosan hydrogel to incorporate 3T3 fibroblasts into their spheroids[886].

Liu et al micro-patterned electrospun fibrous mats for the co-culture of rat hepatocytes, HUVECs, and 3T3 fibroblasts lasting up to 15 days[847]. They observed the formation of hepatocyte

spheroids and noted co-culture with both fibroblasts and endothelial cells enhanced albumin secretion, urea synthesis, and CYP450 expression as compared to monoculture or co-culture with either fibroblasts or endothelial cells alone. While this system incorporates all three cell types on the same culture surface, each cell type is seeded in a specific area, limiting direct contact but allowing paracrine communication[847].

Alternatively, primary rat hepatocytes and 3T3 fibroblasts can initially be co-cultured on 2D micro-patterned surfaces and the resulting cell aggregates detached and encapsulated in PEG to form 3D spheroids[873]. These spheroids show dose-dependent acetaminophen toxicity responses, as well as CYP450 induction by model compounds, such as rifampin and phenobarbital. Additionally, the small size of these spheroids (~100μm) prevents the formation of a necrotic core. However, this system has not been characterized with human hepatocytes.

Bell and colleagues have generated spheroids from primary human cryopreserved hepatocytes and NPCs that demonstrate preserved metabolic function and viability up to five weeks in culture[874]. Beneficially, this system uses all human cells, is highly scalable (made in 96 well plates), and demonstrates long term response to hepatotoxins. However, the cell-to-media ratio is quite low, which may reduce the impact of paracrine factors secreted by the spheroids.

There are two 3D spheroid systems commercially available. First, RegeneTox™ marketed by Regenemed Inc. allows for the co-culture of NPCs and hepatocytes through use of a transwell insert. NPCs are seeded above two interconnected nylon scaffolds and cultured for a week prior to hepatocyte isolation and seeding. The media are changed three times a week and the co-cultures can last more than three months in the system, as demonstrated by albumin, urea, transferrin, and fibrinogen secretion, and stable CYP1A1, 3A4, and 2C9 activity. Moreover, the liver tissue was responsive to inflammatory stimuli by releasing mediators such as TNFα and IL-8[875]. However,

this system is relatively low throughput (24-well plate equivalent) and utilizes a culture surface orders of magnitude stiffer than native liver tissue.

GravityTRAP™ marketed by InSphero AG uses a hanging drop platform to seed cells in a concentrated suspension to grow liver tissue in 3D. These tissues can be transferred into a specialized 96-well plate for culture and analysis, remaining viable for at least 4 weeks. Spheroids demonstrate robust CYP3A4 expression and albumin production compared to 2D cultures. Spheroid size is highly reproducible but may be time consuming without automation. Additionally, spheroid size is limited, as spheroids larger than 200μm develop central necrosis due to hypoxia[876].

While the dimensionality of these systems allow for more complex intercellular communication than 2D culture systems, they do not incorporate flow, which is known to be important for maintaining liver-cell specific functions.

3.7.2 Perfusion Culture Systems

Perfusion culture systems allow for the incorporation of physiological "blood" flow (media), which is an important factor for hepatocyte detoxification of drugs[887] and establishment of the physiological oxygen and chemical gradients. Such systems will be grouped by their size (both volume and cell number), designated as either macroscale or microscale. A summary of the properties of the discussed systems can be found in Table 13.

Table 17. Perfusion hepatocyte culture systems

System	Type	Cell Type(s)	CT (d)	Flow Rate (µL/min)	Volume (mL)	CYP450	Urea	Albumin	Throughput
Spinning Bioreactor Spheroids[888]	Macro Perf	PHH	21-28	41.6	300	Stable	↓	↑	High
Radial Flow Bioreactor[889]	Macro Perf	PRH + 3T3	5-11	830	54	N.D.	↓	↓	Medium
Multichamber Modular Bioreactor[890]	Macro Perf	PHH	7-21	250-500	5	Stable or ↓	Stable	Stable	Low
LiverChip™ by CN Bio Innovations Ltd.[832]	Micro Perf	PHH + HNPC	15-29	60	1.6	Stable	Stable	Stable	High
Metabolomics on a Chip[891]	Micro Perf	HepG2/C3A	2	10	3	Stable	N.D.	Stable	Low
Hollow Fiber Perfusion Bioreactor[892]	Micro Perf	PHH + HNPC	10	3000	0.5	↓	↓	N.D.	Low
Artificial Liver Sinusoid[893,894]	Micro Perf	PHH or PRH or HepG2/C3A	7-8	0.01 – 0.02	0.075	N.D.	N.D.	Stable	Low
HµREL® biochips by HµREL Corp.[867,895]	Micro Perf	cPHH + HNPC	6	4.5	0.1	Stable or ↓	N.D.	N.D.	Medium
Human Liver Sinusoid[896,897]	Micro Perf	PHH & cPHH + EA.hy926 + U937 + LX-2	28	0.08 to 0.25	0.1	Stable	Stable	Stable	Medium

A summary of properties of different perfusion hepatocyte culture systems. Macro = Macroscale; Micro = Microscale; Perf = Perfusion; CT (d) = Culture Time in days; PHH = Primary Human Hepatocyte; cPHH = cryopreserved Primary Human Hepatocyte; PRH = Primary Rat Hepatocyte; 3T3 = 3T3 Fibroblasts; HNPC = Human Non-Parenchymal Cells; N.D = Not determined; EA.hy926 = human endothelial cell line; U937 = human monocyte cell line; LX-2 = human hepatic stellate cell line

3.7.2.1 Macroscale

A few groups have applied large scale perfusion bioreactor devices to the culture of hepatocyte spheroids for drug discovery applications. For example, Tostoes and colleagues demonstrated the culture of primary human hepatocyte spheroids in a spinning perfusion bioreactor for up to 3-4 weeks[888]. The working volume was 300mL and 20% of this was replaced daily through medium perfusion. They demonstrated CYP450 induction and the metabolism of a model substrate, 7-methoxycoumarin. While albumin synthesis increased and stabilized over the culture period, urea synthesis significantly decreased with culture, likely due to spheroid hypoxia. Additionally, this culture system has only been tested for hepatocyte monoculture.

Another system uses a radial flow bioreactor (RFB), where the bed of matrix housing cells separates the media inlet and outlet, which facilitates nutrient and oxygen diffusion across the tissue. Park and colleagues co-cultured rat hepatocytes and 3T3-J2 fibroblasts on a series of stacked glass substrates, with microfabricated grooves and a collagen coating, to improve oxygen delivery to the tissue[889]. However, while RFBs culture large number of hepatocytes ($2\text{-}5x10^7$), the high flow rates used cause loss of hepatic-specific functions after a few days due to unphysiological shear stresses[889].

Vinci and colleagues cultured primary human hepatocytes on coverslips for 7 days then transferred the coverslips into either their multichamber bioreactor or a new dish for continued static culture. They found that the expression of metabolic (CYP450) and transport (OATP, MDR1) genes were much higher under perfusion conditions and that higher flow rates further enhanced expression[890]. The use of primary human hepatocytes makes this system amenable to investigating human-specific drug interactions. However, the working volume (5mL) is quite large compared to the cell seeding number (200k), which reduces the influence of secreted factors.

While macroscale perfusion culture systems allow for the influences of shear stress and nutrient supply on drug detoxification to be extrapolated, they carry several weaknesses. First, the number of cells required is substantially higher than smaller-scale culture systems, which may prove challenging given the limited availability of hepatocytes. Second, the media volume to cell volume ratio is much higher than is seen *in vivo*. Third, many of these systems replace the circulating media with fresh media on a continuous basis. As such, these systems may demonstrate reduced signaling from soluble factors released by the hepatic tissue.

3.7.2.2 Microscale

In contrast to macroscale (5-300mL) perfusion culture systems, the use of microscale (0.1-3mL) systems allows for higher cell-to-media volume ratios, which approach those seen *in vivo*. The LiverChip by CNBio Innovations (originally Zyoxel) models the liver tissue with a perfusion system on a larger volume scale than most micro-perfusion systems (1.6mL)[751,753,832,898]. Earlier generations of this system utilized alternative well geometry[899,900]. Hepatocytes and a full complement of donor matched NPCs are seeded on polystyrene (mechanically stiff) scaffolds at a 1:1 ratio. Fluid flow is driven by pneumatic pumps at a rate of 60 µL/min through the scaffolds, providing direct, but physiologically representative shear stresses on the resident tissue cells (Figure 9). Tissues are established and remain functional up to one month, as measured by blood urea nitrogen levels, fibrinogen, alpha-1 antitrypsin (A1AT), and CYP450 activity. The culture chamber allows for a 3D hepatic tissue to form, which provides an advantage over 2D culture systems by facilitating cell junction formation. This culture system has been used to study metastasis of cancer cells in the context of the liver microenvironment[751,753,832,898] and probe the influence of LPS on hydrocortisone metabolism[858]. This system offers a unique opportunity to

study cancer drug efficacy and hepatotoxic effects in a single system. However, there is currently no effective way to physically monitor tissue formation during the culture period.

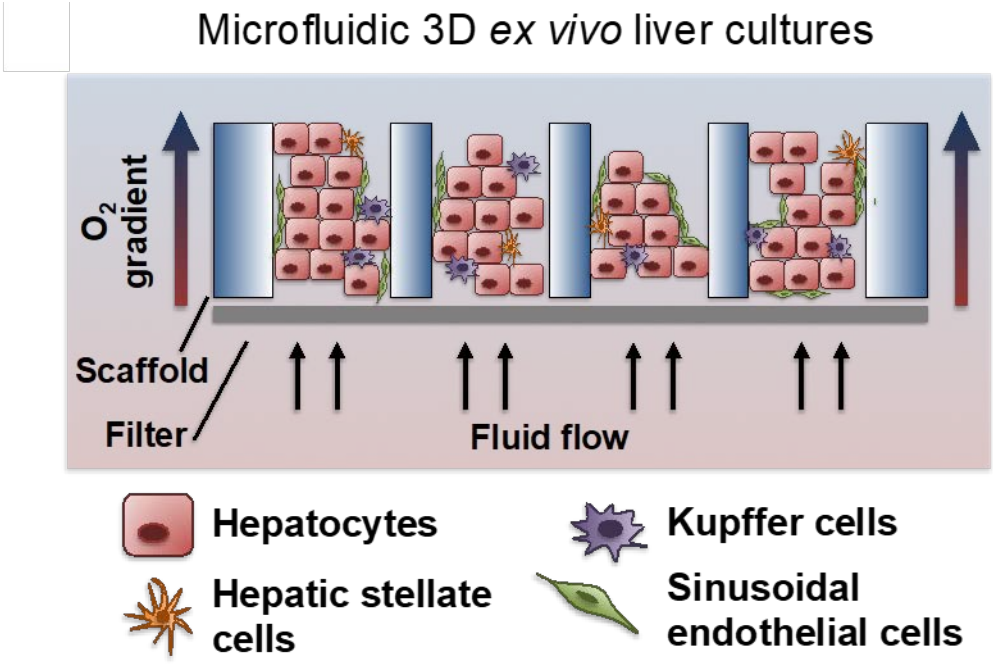


Figure 11. A schematic of the LiverChip by CN Bio Innovations Ltd.

Three dimensional liver tissues are subject to fluid flow in micro-scale pores of a scaffold, which facilitates shear stresses and establishment of a physiological oxygen gradient.

Choucha-Snouber and colleagues designed a liver biochip to investigate metabolic responses to the anticancer drug flutamide[901]. Their chip is fabricated from PDMS and contains fibronectin-coated microstructures for cell seeding. HepG2/C3a cells are seeded in the system and kept in culture for a total of 48 hours, 24 hours of which is for perfusion and drug treatment. In comparison to the LiverChip, this system uses a lower flow rate of 10µL/min. The low volume of the biochip (40µL) is desirable to achieve a physiologic ratio of media supply to cell volume.

However, this system has not tested co-culture with NPCs. Additionally, using PDMS as the fabrication substrate allows for gasses to readily equilibrate between the external environment and the cell culture chamber, which may limit modeling of a physiologically hypoxic liver microenvironment. Additionally, small hydrophobic compounds adsorb to PDMS, which limits the drugs this system can accurately assess without extensive calibration and testing. This design was extended to investigate kidney-liver co-culture systems[891].

Hoffmann and colleagues designed a hollow-fiber based microscale perfusion culture system, with a volume of 0.5mL[892]. A unique feature of this system is the incorporation of three different supply channels to the cultured tissue – two media circuits and one gas circuit. The authors demonstrate robust tissue formation, which includes NPCs. Moreover, NPCs were noted to form vascular-like structures. While the culture volume is low, the flow rate and fresh media replenishment rate are quite high in comparison (3mL/min and 0.5mL/hour respectively). This may prove a limiting factor for analyzing long-term effects of secreted factors. Additionally, the expression and activity of several CYP450 (3A4 and 2B6) isoforms decreases dramatically between days 3 and 10 of culture[892]. Since 50% of currently tested drugs are metabolized by CYP3A4[902], the decrease in its activity over the culture period may make this system not suitable for long term toxicity or metabolic assays.

CellAsic designed a hepatic sinusoid cord-like culture system, in which a central cord of cells is enveloped by a fluid transport channel. Communication between the cells and fluid channel was accomplished by small (1µm by 2µm) canals, limiting transport to diffusion. The system is primed with fluid and cells are subsequently seeded. After cell seeding, the chip is placed in the incubator on an incline to facilitate gravity-driven continuous flow, at rates of 10-20nL/min, to supply the tissue. The system can maintain hepatocytes for over 1 week, as determined by albumin

secretion, and exhibit dose-dependent hepatotoxicity of diclofenac as seen *in vivo*[893,894]. Thirty two independent culture systems can be run simultaneously on a device the size of a 96-well plate. While this system generates 3D tissue, an improvement over 2D culture systems, it has not been investigated for culture periods longer than 7 days.

HμREL® biochips by Hurel Corp. allow up to 8 microfluidic circuits to operate in parallel[895]. These chips contain two cell culture compartments connected in series, one of which was designed to house liver tissue. Cells are seeded onto the polystyrene biochip either in monoculture or co-culture, using human hepatocytes and NPCs. Flow was generated by use of a peristaltic pump with a flow rate of 4.5μL/min/chip. Hepatocyte co-cultures in the system subjected to flow demonstrated increased metabolic activity when compared to static monocultures or co-cultures and remained viable for up to 6 days[867]. One advantage of this system is that the HμREL tubing exhibits limited adsorption of hydrophobic drug molecules. Additionally, the polystyrene housing reduces gas permeability of the device, facilitating formation of a hypoxic microenvironment. However, the tissues formed in this system are not 3D and the maximum culture period is limited compared to other systems. Moreover, CYP450 isoforms exhibit varying activities in the system – while CYP3A4 activity increases over the 6 day culture duration, CYP2D6 activity decreases after 2 days of culture[867].

Finally, the liver acinus microphysiology system (LAMPS) is a microfluidic platform to investigate hepatic physiology, drug safety, and disease models[759] that evolved from the first generation liver MPS (SQL-SAL)[896]. The LAMPS incorporates either fresh or cryopreserved human hepatocytes, as well as a full complement of non-parenchymal cells: human endothelial (primary or TMNK-1), immune (THP-1 or U937), and stellate (LX-2) cells in a Nortis, Inc microfluidic device. A porcine liver extracellular matrix (LECM), ca. 10μm thick, is layered

between the endothelial cells and the hepatocytes. Approximately 20% of the hepatocytes and stellate cells contain fluorescent biosensors that measure apoptosis and reactive oxygen species for real-time fluorescence readouts[903], along with analysis of the secretome and metabolic activity. Distinct zone 1 and zone 3 microenvironments are created that allow the direct determination of zone-specific physiological and disease functions[904]. This system is viable for at least 28 days under continuous perfusion flow and exhibits concentration- and time-dependent toxicity profiles to drugs (e.g. troglitazone) in a similar manner to that seen clinically[905]. Structurally, the endothelial cells are separated from the hepatocytes by a biomimetic of the Space of Disse forming a three-dimensional layer of cells and LECM. The immune cells permit the system to be LPS responsive, releasing TNFα upon stimulation and in combination with some drugs causes immune-mediated hepatotoxicity. Moreover, the LX-2 cells can be activated in response to methotrexate treatment, resulting in the expression of smooth muscle actin (SMA) and the production of collagen 1A2, reflecting a fibrosis phenotype. Most importantly, this system is amenable to real-time transmitted light and fluorescence imaging, allowing for investigating toxicity kinetics and disease progression.

The platform is continually being evolved to replace the NPCs with either primary human cells or iPSC-derived cells, since the use of immortalized NPCs may not reflect full physiological capabilities. This platform contains PDMS which requires the presence of carrier proteins for hydrophobic drugs and pre-measurement of the loss of drugs to the device before making any interpretations[897]. While the media in this system is not recirculated in the current format, the small volumes and low flow rates allow the detection of paracrine signaling mediators secreted by the cells. Although, there are other advantages to having the media recirculated (see above).

3.7.3 Conclusions and Future Directions

Advanced liver culture systems hold the potential to transform drug discovery and development, through more accurate modeling of human *in vivo* pharmacokinetics and pharmacodynamics, as well as understanding mechanisms of disease progression and toxicity. Several factors affect the accuracy of these model systems in predicting pharmaceutical toxicity. The cells used in the culture system and the culture surface (e.g. plastic, hydrogel) allow for modeling cell-cell and cell-ECM interactions respectively. Systems that include media flow ("blood flow") provide mechanical stimuli that benefit tissue function. Inclusion of cells derived from human tissues allows for human-specific pathways to be modeled. Special attention should be paid to the use of primary versus immortalized cells, as the latter may elicit signals not seen in physiologic conditions. The use of animal cells instead of human cells may encounter similar intractable weaknesses. Importantly, the expression levels of CYP450 isoforms and drug transporters over the culture period may influence toxicity predictions.

Future directions for improving these systems include incorporating lentiviral-induced fluorescent biosensors for an automated readout of various cellular functions[903] and finer architectural control of liver microstructure. Importantly, the ability to image the devices in real time is highly advantageous. Fabrication of devices using materials other than PDMS – which adsorbs hydrophobic molecules and biologics – will improve the utility of these devices as pharmacokinetic models for drug discovery. Moreover, procurement of a universal, readily available cell source (potentially iPSC-derived hepatocytes) will improve the ease with which these systems are used for drug characterization studies. Networking of additional organ chips, such as gut and kidney, will contribute additional signals to the liver tissue, allow for modeling of drug absorption, and disposition after hepatic metabolism. Finally, further development and usage

of quantitative systems pharmacology as a new paradigm in drug discovery and development involving the integration and iteration of experimental and computational models, in parallel with advanced culture systems will facilitate complex data analysis and may allow for predictive models to be generated[906,907].

It is hoped that the development of validated human experimental model systems can be used in place of animal models to improve preclinical drug selection. By implementing more accurate human experimental model systems, it is expected that there will be increased efficacy and safety in clinical trials leading to improved therapeutics at a lower cost.

4.0 RATIONALE AND HYPOTHESES

Developing or repurposing novel therapies to serve as long term adjuvants for women with a history of breast cancer is necessary in order to prevent recurrence. Statins have been shown in both epidemiological and experimental studies to have anti-tumor effects, yet the mechanisms and factors that make tumor cells more or less susceptible to statin therapy remain unclear. While statins have a benefit on both breast cancer recurrence and breast cancer specific mortality, no difference in incidence of primary cancer is noted with statin usage.

Previous work has suggested the anti-tumor effects of statins result from a decreased abundance of prenylating intermediates, which reduce activity of signaling g-proteins that normally promote growth and survival in neoplastic cells. An example protein negatively impacted by lack of prenylation is Ras, whose membrane localization is critical to its signaling function. Our lab and others have shown that tumor dormancy is marked by a state of cellular quiescence, in which cells are arrested in G0/G1. One of the contributing factors to this quiescence is the growth suppressive effect of E-cadherin, which is re-expressed upon metastatic seeding and entry into dormancy. The microenvironment is abundant with growth factors and other mitotic stimuli, which can provoke dormant tumor cells to undergo a secondary EMT and outgrow.

As tumor cells emerging from dormancy experience enhanced flux through pro-mitotic and pro-migratory signaling pathways, such as PI3K-Akt and MAPK, it is likely they are more susceptible to the anti-tumor effects of statins. These findings lead us to the central hypothesis of this work: **statins act by selectively targeting dormant micrometastases that are primed to outgrow, keeping them suppressed in a state of quiescence and reducing breast cancer recurrence and mortality (Figure 12)**.

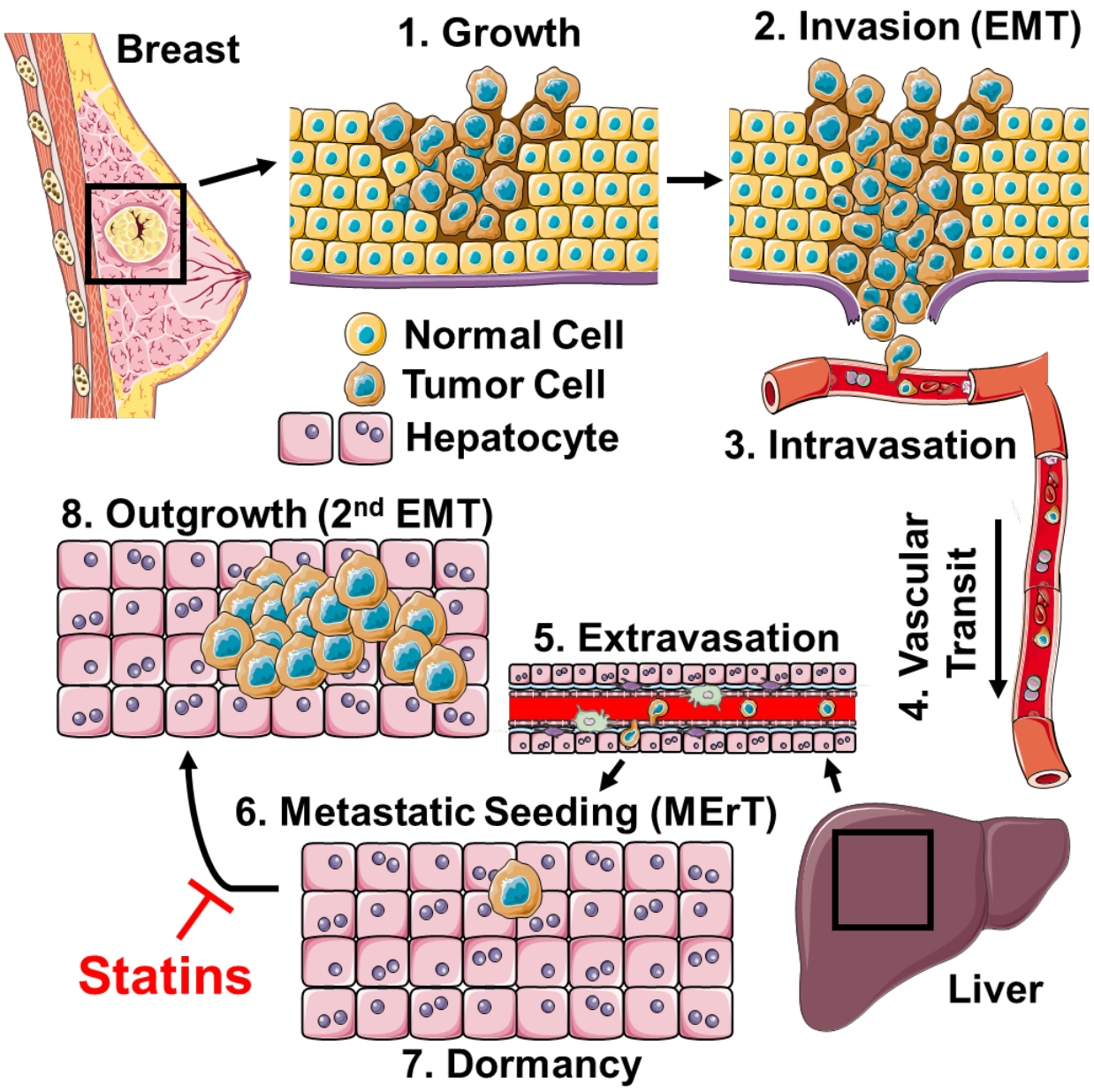


Figure 12. Proposed influence of statins on cancer mortality

Statins block tumor cell emergence from dormancy by decreasing the proliferation of micrometastases at the metastatic site. This results in a decrease in cancer recurrence and mortality. EMT = epithelial-to-mesenchymal transition; MErT = mesenchymal-to-epithelial reverting transition. This diagram was created using images from Servier Medical Art[46].

5.0 STATIN-INDUCED MEVALONATE PATHWAY INHIBITION ATTENUATES THE GROWTH OF MESENCHYMAL-LIKE CANCER CELLS THAT LACK FUNCTIONAL E-CADHERIN MEDIATED CELL COHESION

5.1 PROLOGUE

The first piece of work contributing to this thesis was published in Scientific Reports at the end of 2014[2], which resulted mainly from work I conducted on the manuscript during my summer rotation in collaboration with two Japanese students working with Dr. Oltvai, the senior author on the study. The majority of this chapter is previously published in that manuscript, and the corresponding reference can be found both at the beginning of this document and in the references section. Of note, the "Primary Data" section refers to a discussion of the data published in the body of the manuscript, the "Supplemental Data" refers to data published in the supplement of the manuscript, and the "Supporting Data" refers to unpublished data that support the conclusions of this study.

In this work, we investigated the growth suppressive effects of atorvastatin, a model lipophilic statin, on a panel of cancer cell lines. We confirmed that atorvastatin sensitivity was marked by a decrease in total intracellular cholesterol levels and that sensitive cell lines could be rescued from these effects upon addition of mevalonate, the product of the inhibited HMGCR enzyme. We demonstrate that susceptibility to atorvastatin-mediated growth suppression is not due to differing protein levels of the target enzyme, as all cell lines tested expressed comparable levels. Moreover, we find that all statin resistant cell lines strongly express E-cadherin on the

membrane and that expression of E-cadherin on the membrane in cells with normally no expression is sufficient to induce resistance to atorvastatin.

These data are a significant foundation to this body of work for several reasons. First, this manuscript demonstrates direct growth suppression of tumor cells by atorvastatin by looking at growth kinetics. Most studies in the literature demonstrate growth suppression or cellular death by investigating one specific experimental time point with cytotoxicity assays. Second, this study demonstrates that statins act to suppress cancer cell growth by the same mechanism as they act to reduce cholesterol, namely by suppressing HMGCR. Finally, this study uncovers E-cadherin as both a biomarker and mechanism of resistance to statin therapy. Given the overarching hypothesis of this thesis involves the ability of statins to target metastatic-emergent breast cancer to reduce recurrence and mortality, these data support this theory by suggesting statins are the most effective against the cells that have undergone a secondary EMT to emerge from dormancy.

5.2 MATERIALS AND METHODS

Cell cultures. Fourteen cell lines from the NCI-60 cancer cell panel—colon cancer (HCT-116 and KM-12), ovarian cancer (IGROV1 and OVCAR3), breast cancer (HS-578T and T47D), lung cancer (HOP-92 and NCI-H322M), prostate cancer (PC-3 and DU-145), melanoma (SK-MEL-5 and MDA-MB-435), and brain cancer (SF-295 and SF-539)—were cultured in RPMI 1640 medium (Life Technologies, Grand Island, NY), supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS, Life Technologies) and 1% penicillin/streptomycin (Life Technologies) at 37°C with 5% CO_2.

Atorvastatin treatment and cell proliferation assay. Atorvastatin (Sigma-Aldrich, St. Louis, MO) at a final concentration of 10 μM was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich; final concentration of 0.1% in RPMI 1640 medium). The cells were seeded in 6-well plates at a density of 1×10^5 cells/ml and incubated overnight prior to treating with 10 μM atorvastatin or 0.1% DMSO, which served as a control. Cell proliferation was quantified at 2, 4, and 6 days by direct cell counting with Scepter™ Handheld Automated Cell Counter (EMD Millipore, Billerica, MA) using Scepter™ Tips–60 μm sensor (EMD Millipore), or by crystal violet staining. Preliminary studies demonstrated that the two methods yielded congruent results.

Mevalonic acid treatment in atorvastatin sensitive cells. To determine whether mevalonic acid treatment reverted the atorvastatin-sensitive phenotype, the cell lines whose proliferation was inhibited by atorvastatin were seeded in 6-well plates at a density of 1×10^5 cells/ml, incubated overnight, and then treated with 10 μM atorvastatin and various concentrations (25 μM, 50 μM, 100 μM, and 200 μM) of R-mevalonic acid (Sigma-Aldrich) for 24 hours. These cells were also photographed with a phase-contrast microscope to capture any morphological changes.

Cholesterol staining. Subconfluent (40–60% confluency) cells grown on coverslips in a 24-well plate were incubated with RPMI 1640 medium supplemented with 10% HI-FBS, 10 μM atorvastatin, and a cholesterol intercellular trafficking inhibitor, 1.25 μM U18666A (Cayman Chemical, Ann Arbor, MI). Some of the atorvastatin sensitive cells (HOP-92 and PC-3) were also treated with 100 μM R-mevalonic acid (Sigma-Aldrich). Cells treated with 0.1% DMSO and 1.25 μM U18666A served as control. After 24-hours incubation, the cells were stained using the cholesterol cell-based detection assay kit (Cayman Chemical) according to the manufacturer's instructions. Briefly, the cells were fixed with 4% formaldehyde in Tris-buffered saline (TBS) for 10 min

and then washed in TBS with 0.1% Tween 20 (TBS-T). The cells were incubated with 50 μg/ml Filipin III in TBS, a probe used for the fluorescent detection of sterols (excitation/ emission, 340–380/385–470 nm). After 1 hour incubation with Filipin III in the dark at room temperature (RT), the cells were washed in TBS-T, followed by detachment and mounting of the coverslips in the aqueous-based mounting medium (Clearmount™, Invitrogen, Carlsbad, CA). Images were taken under an Olympus Provis fluorescence microscope (Olympus Optical, Tokyo, Japan) equipped with a 40X oil objective lens. Quantification of the cholesterol levels was accomplished using the Granularity algorithm in the MetaXpress image analysis software (Molecular Devices Corp., Sunnyvale CA). The total integrated granule intensity was measured for each image and the percent cholesterol remaining after atorvastatin treatment was calculated as the ratio of granule intensity in the atorvastatin cells to that of the DMSO treated cells.

Western blotting. After removing spent media and washing cells with cold phosphate buffered saline (PBS), the cells were incubated with cold Pierce RIPA lysis buffer (Thermo Scientific, Hudson, NH) containing Halt™ Protease Inhibitor Single-Use Cocktail, Halt™ Phosphatase Inhibitor Single-Use Cocktail (Thermo Scientific) and 1 mM dithiothreitol (DTT) for 15 min with occasional swirling. Then, the cells were scraped, homogenized with a 26-gauge needle and vortexed at the highest setting for 1 min; the lysates were cleared by centrifuging at 16,000g at 4 ºC for 15 min. Protein concentration was determined with the bicinchoninic acid (BCA) method (BCA Protein Assay - Reducing Agent Compatible; Thermo Scientific). Proteins were separated on NuPAGE 4–12% Bis Tris gel electrophoresis (Life Technologies), and transferred to a nitrocellulose membrane (iBlot Gel Transfer Stacks Nitrocellulose; Life Technologies) using iBlot Gel Transfer Device (Life Technologies). The membrane was probed with monoclonal rabbit antibodies, anti-HMGCR (1:500, Abcam Inc., Cambridge, MA), anti-

vimentin (1:1000, Abcam), and anti-E-cadherin (1:1000, Cell Signaling Technology, Beverly, MA). A monoclonal mouse antibody to β-actin (1:500, Abcam) was used as a loading control. Immunodetection was performed using the iBlot Western Detection chromogenic kit (Life Technologies).

Immunofluorescence microscopy. Cultured cells grown on coverslips in a 24-well plate were fixed with 2% paraformaldehyde (Sigma-Aldrich) for 30 min, washed in PBS, and then permeabilized with 0.1% Triton-X-100 (Fisher Scientific, Pittsburgh, PA) made in PBS for 15 min. Following a PBS wash, non-specific proteins were blocked in 2% BSA for 15 min at RT. The cells were incubated with a mixture of two primary antibodies: monoclonal rabbit antibody to vimentin (1:100, Abcam) and monoclonal mouse antibody to E-cadherin (1:50, Abcam) in a humidified atmosphere for 1 hour at 37°C. Coverslips were then probed with Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 555 goat anti-rabbit IgG (both; 1:200, Abcam) in the dark for 15 min at RT. Following a PBS wash, nuclei were stained with Hoechst 33342 (50 µg/ml) for 5 min at RT, washed and mounted in an aqueous-based mounting medium Clearmount^{TM} (Invitrogen). Images were captured with the 40X oil objective lens on the Olympus Provis fluorescence microscope (Olympus Optical).

IC_{50} determination. MDA-MB-231 (breast cancer) and PC-3 (prostate cancer) cell lines were seeded in 12-well plates at a concentration of 1×10^5 cells/ml. The next day, cells were treated with 0.1 µM, 0.3 µM, 1 µM, 3 µM, 10 µM, or 30 µM atorvastatin. Cells treated with 0.3% DMSO (the concentration of DMSO in the 30 µM atorvastatin treatment condition) served as a control group. Three days after treatment, the cells were washed with PBS and fixed in 4% formaldehyde (F79-1, Fisher Scientific) for 15 minutes. The cells were washed with milli-Q water and stained with 0.5% w/v crystal violet (Sigma-Aldrich) for 10 minutes, and excess dye was washed

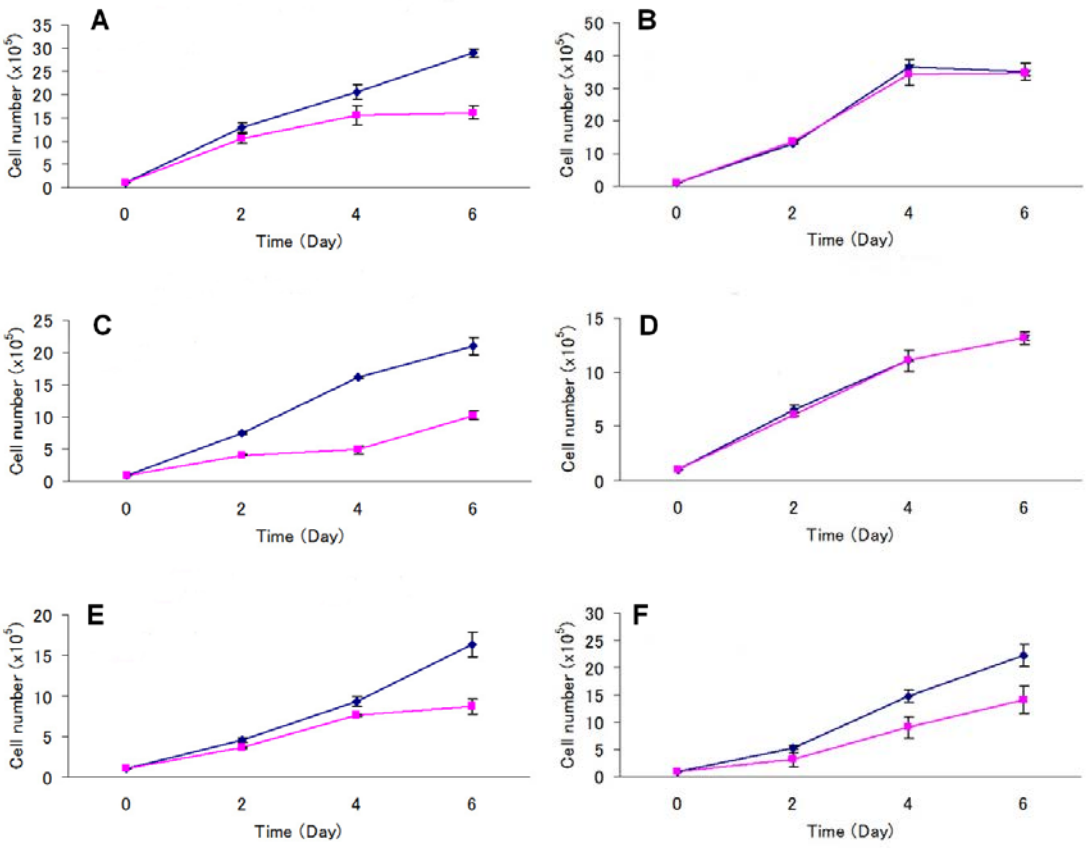
extensively with tap water. The absorbed dye was released with 2% SDS. The supernatants were mixed thoroughly before transferring to a 96-well plate to be read at 560 nm using a Tecan SpectraFluor microplate reader (Tecan US, Durham, NC). IC_{50} values were determined by fitting a standard, four-parameter sigmoid curve to the data. All treatments were carried out in triplicate samples.

5.3 PRIMARY DATA

5.3.1 Variable Growth Inhibition of Cancer Cell Lines with Atorvastatin Treatment

Previous experiments have demonstrated that statins, including atorvastatin (Lipitor), inhibit the growth of a subset of the NCI-60 cancer cell lines, and if one statin inhibited the proliferation of a given cell line, then the other statins also showed similar half maximal inhibitory concentration (IC_{50}) values[908]. To confirm these results, we cultured two cell lines from each of seven organ types obtained from the NCI-60 collection in standard growth medium with 10μM atorvastatin. We found that atorvastatin affected the proliferation rates of these cancer cell lines differentially: the proliferation of some cell lines were fully or partially inhibited by atorvastatin while others were insensitive to it (Figure 13). Some cell lines derived from the same organ type showed differential susceptibility to atorvastatin. For example, HOP-92 and NCI-H322M are both lung carcinoma derived cell lines yet the former is sensitive to atorvastatin whereas the latter is resistant to atorvastatin (Figure 13G, H). Similarly, PC-3 and DU-145 cells are both derived from metastatic prostate tumors of bone and brain respectively[909], yet the former is sensitive to atorvastatin whereas the latter is resistant (Figure 13I, J). In contrast, some cell line pairs from the same organ type

exhibit similar responses to atorvastatin. For example, both SF-295 and SF-539, glioblastoma and gliosarcoma cell lines respectively, exhibit profound atorvastatin sensitivity, even demonstrating possible cytotoxic effects that can be seen starting at 4 days post-atorvastatin treatment (Figure 13M, N). These data demonstrate that atorvastatin can directly suppress tumor cell growth and that the degree of suppression cannot be determined solely from the tumor site of origin.



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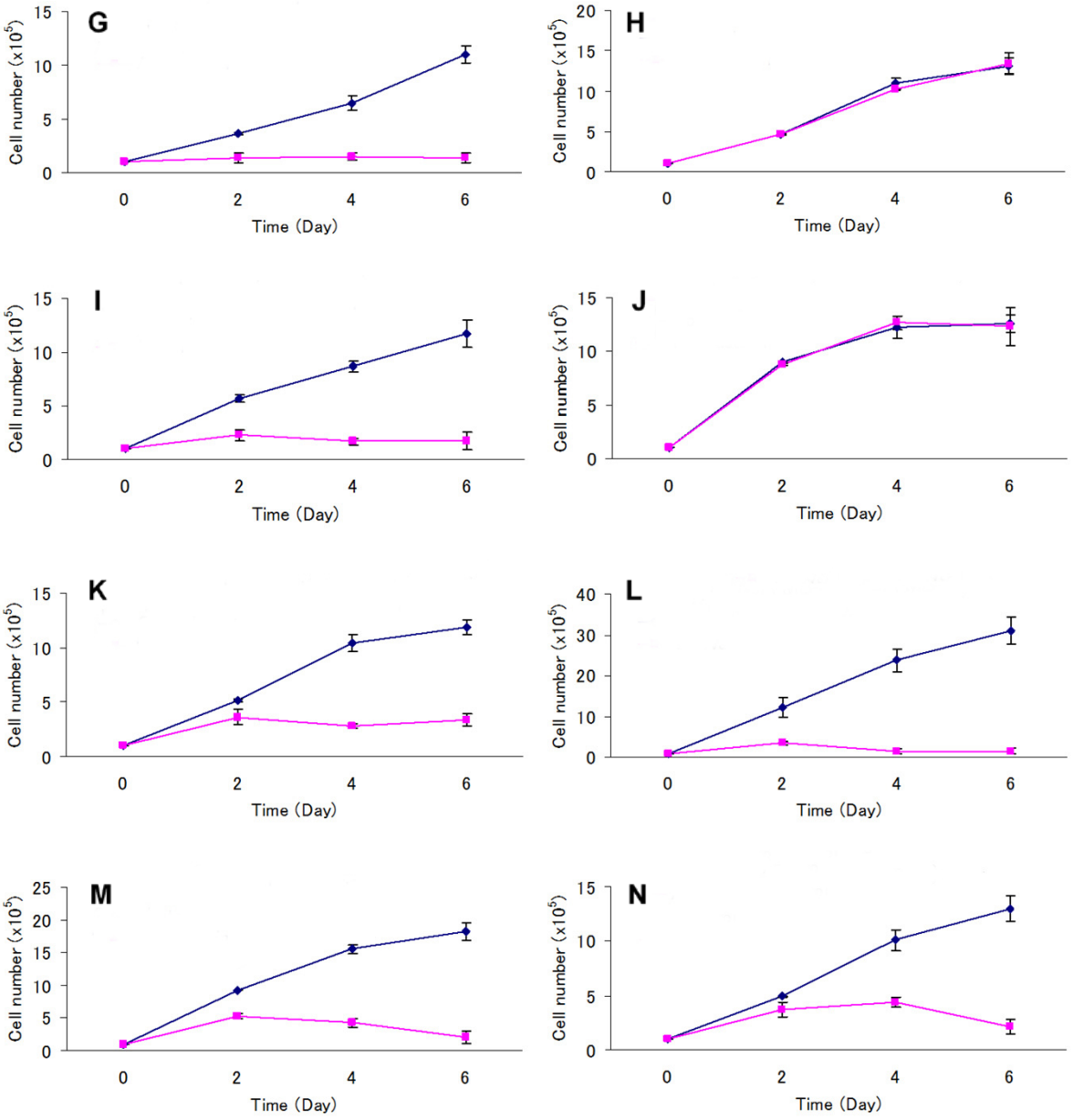


Figure 13. Growth rate of atorvastatin treated NCI-60 cancer cell lines

Colon cancer (A. HCT-116 and B. KM-12), ovarian cancer (C. IGROV1 and D. OVCAR3), breast cancer (E. HS-578T and F. T47D), lung cancer (G. HOP-92 and H. NCI-H322M), prostate cancer (I. PC-3 and J. DU-145), melanoma (K. SK-MEL-5 and L. MDA-MB-435), and brain cancer (M. SF-295 and N. SF-539) cell lines from the NCI-60 cancer cell line collection were treated with 10 μM atorvastatin (purple line) or DMSO vehicle control (blue line), and cell proliferation was quantified at 2, 4, and 6 days by direct cell counting.

5.3.2 Atorvastatin sensitivity correlates with decreased cholesterol levels in atorvastatin-treated cells

Atorvastatin inhibits the enzymatic activity of HMGCR, thus blocking the synthesis of mevalonate and its downstream products that include cholesterol (Figure 11). To directly test the relationship between atorvastatin sensitivity and cholesterol levels, we measured the cholesterol content of the cell lines in the presence or absence of atorvastatin using Filipin III, a polyene macrolide routinely used to assess intracellular cholesterol levels[910]. Representative results are shown in Figure 14. Atorvastatin treatment for 24 hours reduced the levels of intracellular cholesterol in atorvastatin sensitive HOP-92 cells when compared to that of the control DMSO treatment by more than 75% (Figure 14G-I). In contrast, addition of the drug did not affect the levels of intracellular cholesterol in atorvastatin resistant DU-145 (Figure 14A-C). The partially sensitive T47D cell lines showed an intermediate effect on cholesterol after atorvastatin treatment, with a reduction in intracellular cholesterol of almost 20% (Figure 14D-F). Interestingly, while the cholesterol localization does not appear to change with atorvastatin treatment in resistant or partially sensitive cell lines (Figure 14A-F), atorvastatin sensitive HOP-92 see a dramatic reduction in perinuclear cholesterol accumulation.

It is possible the decreased density of cholesterol near the nucleus is due to a dramatic reduction in total cholesterol, yet the cholesterol appears more diffusely distributed after atorvastatin treatment. The complete data set for all fourteen cell lines is shown and discussed in the supplemental information section of this chapter (Figure 21). Thus, the sensitivity of cell lines to atorvastatin-induced growth inhibition inversely correlated with the intracellular cholesterol levels following atorvastatin treatment. These data suggest that sensitivity to statin-mediated growth suppression is directly proportional to the degree of shut down of the mevalonate pathway,

as the cells most greatly affected by growth suppression also demonstrate that most profound reductions in the end product of that pathway (cholesterol).

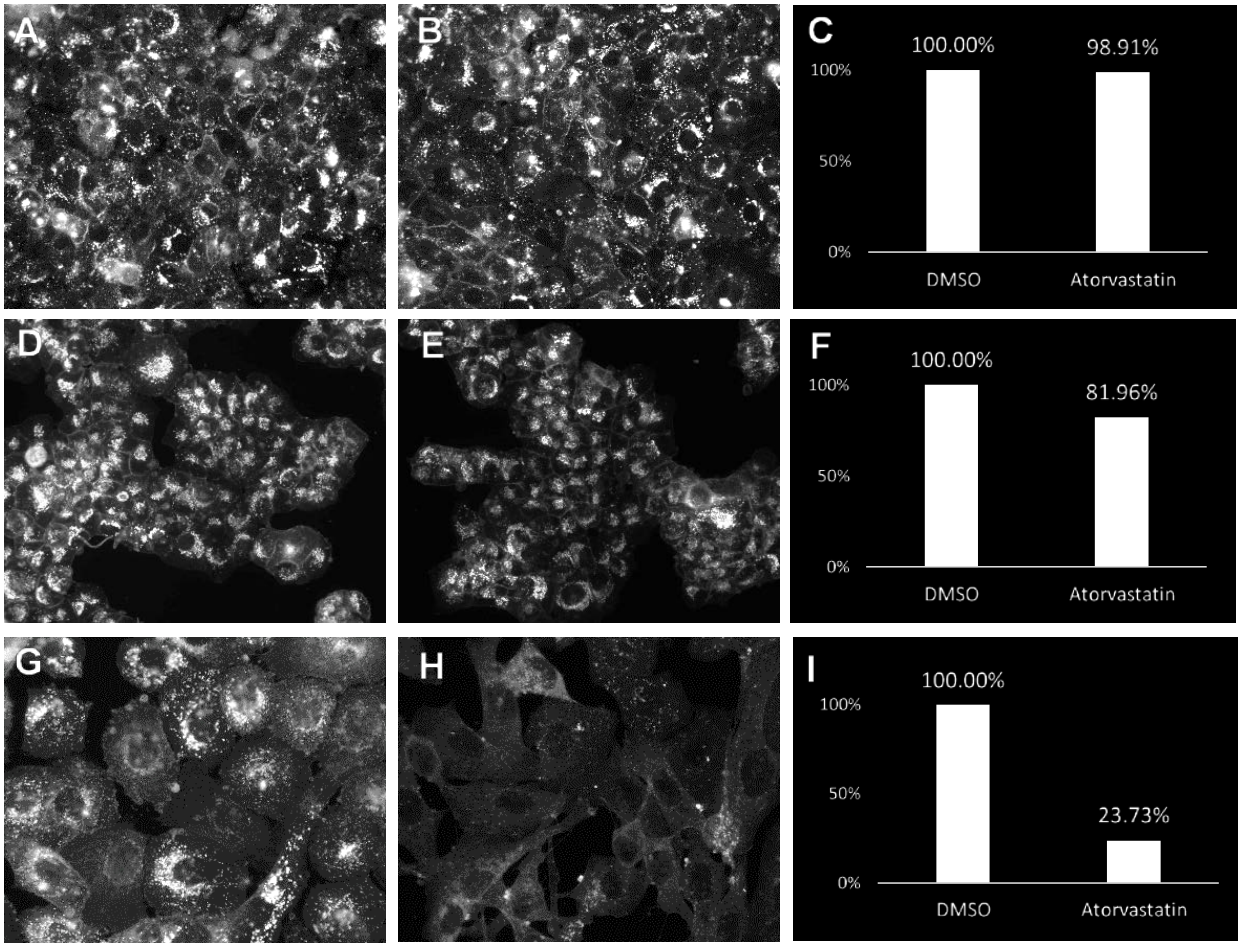


Figure 14. Cholesterol content of atorvastatin treated NCI-60 cancer cell lines

Cell lines were treated with 10μM atorvastatin or 0.1% DMSO for 24 hours. Intracellular cholesterol levels were determined by Filipin III staining. Results for (A-C) atorvastatin resistant (DU-145), (D-F) partially sensitive (T47D), and (G-I) sensitive cell lines (HOP-92) are shown. (A, C, F) DMSO control; (B, D, F) 10μM atorvastatin treated cells; (C, F, I) histogram of cholesterol levels normalized to the DMSO controls.

5.3.3 Total vimentin and E-cadherin expression are not suitable markers for statin sensitivity

The differential sensitivity of tumor cell lines to atorvastatin could be utilized for adjuvant cancer therapy if highly sensitive biomarkers were identified. Our previous analysis of the NCI-60 cancer cell lines indicated that cells with larger diameters in culture are more sensitive to statin treatment. Furthermore, increased cell size in culture correlated with higher vimentin protein expression, a standard marker of mesenchymal cell lineage, while the epithelial marker E-cadherin negatively correlated with increased cell size[911]. As these two markers are routinely used in standard immunohistochemistry of tumor biopsy specimens, we probed their utility as biomarkers of atorvastatin sensitivity.

First, we performed Western blot analyses to determine the average vimentin and E-cadherin expression in the fourteen cell lines. We found that several cell lines express vimentin with absent or minimal E-cadherin expression while cell lines deficient in vimentin expressed a medium to high level of E-cadherin (Figure 15). Of note, some cell lines expressed both vimentin and E-cadherin (Figure 15); however, they also exhibited highly variable atorvastatin sensitivity. For example, the PC-3 and DU-145 prostate cancer cell lines express both proteins at comparable levels (Figure 15), yet their responses to atorvastatin are completely opposite (Figure 13). These data indicate that the correlation between the total expression levels of the mesenchymal marker vimentin and epithelial marker E-cadherin expression cannot be used as dependable biomarkers for predicting or identifying atorvastatin sensitive cancer cells. These data are significant as often changes in cancer cell phenotype that accompany EMT are determined by analysis of total levels of E-cadherin[45], which suggest alternative analyses or markers are needed to determine susceptibility to statin therapy.

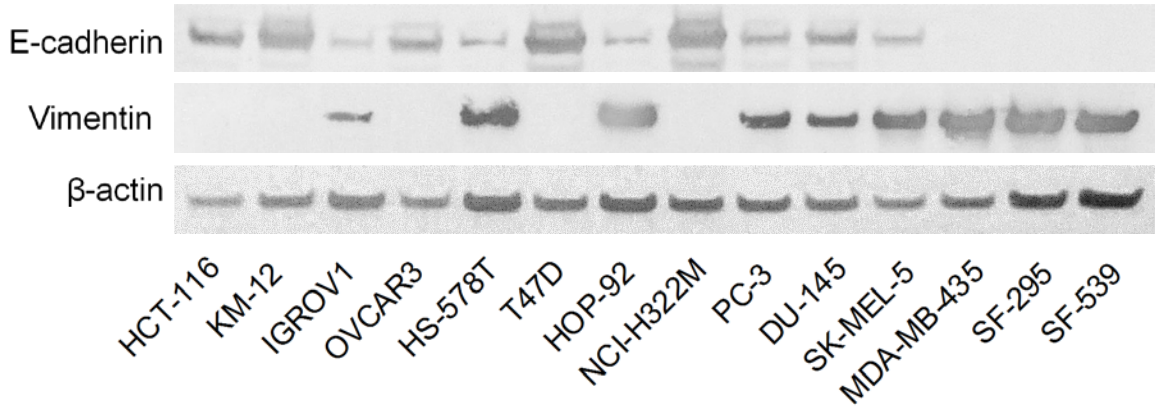


Figure 15. E-cadherin and vimentin expression of NCI-60 cancer cell lines

The expression levels of E-cadherin and vimentin in the indicated fourteen NCI-60 cell lines were determined by western blotting. Colon cancer (HCT-116 and KM-12), ovarian cancer (IGROV1 and OVCAR3), breast cancer (HS-578T and T47D), lung cancer (HOP-92 and NCI-H322M), prostate cancer (PC-3 and DU-145), melanoma (SK-MEL-5 and MDA-MB-435), and brain cancer (SF-295 and SF-539). β-actin expression was used as the loading control.

5.3.4 Membrane E-cadherin expression is a potential biomarker for atorvastatin sensitivity

While vimentin is a cytoplasmic protein, E-cadherin can be found in different subcellular localizations within tumor cells[912,913]. E-cadherin homodimerization at the cell membrane provides survival signals whereas destabilized E-cadherin molecules are internalized and fail to signal[748]. To uncover the potential differences in E-cadherin localization in the fourteen cell lines, we performed double immunostaining for vimentin and E-cadherin expression, and imaged using immunofluorescence microscopy.

Based on the collected data, the fourteen cell lines were segregated into one of three groups, each with different patterns of vimentin and E-cadherin expression (Figure 16). First, six cell lines without cell surface E-cadherin expression but uniformly expressed cytoplasmic vimentin were atorvastatin-sensitive (Figure 16A-F). In contrast, cell lines that displayed any cell membrane E-cadherin expression were either fully or partially atorvastatin-resistant (Figure 16H-N), irrespective of their cytoplasmic vimentin expression. The degree of vimentin and E-cadherin expression varied among the individual cell lines. For example, DU-145 cells display both cytoplasmic vimentin and heterogeneous cell membrane E-cadherin expression (Figure 16K), whereas OVCAR3 cells are vimentin negative but exhibit both cell membrane and intracellular E-cadherin localization (Figure 16M). Despite this difference, both cell lines are fully resistant to atorvastatin (Figure 13).

A similar variation exists among partially resistant cell lines. T47D and HCT-116 cells are positive for cell membrane and cytoplasmic E-cadherin but are vimentin negative (Figure 16H, I), whereas IGROV1 cell line is comprised of at least two different cell types. A subset of IGROV1 cells are vimentin negative and positive for cell membrane E-cadherin while another subset of cells is positive for cytoplasmic vimentin, and exhibit cell membrane and cytoplasmic E-cadherin (Figure 16J). The only exception is the HS-578T breast cancer cell line that is only partially atorvastatin sensitive (Figure 13), even though it has similar vimentin and E-cadherin expression pattern as that observed in atorvastatin-sensitive cell lines (Figure 16G). These results suggest that cell membrane E-cadherin expression is a marker of full or partial atorvastatin resistance, whereas uniform vimentin expression without any cell membrane E-cadherin expression largely correlates with atorvastatin sensitivity.

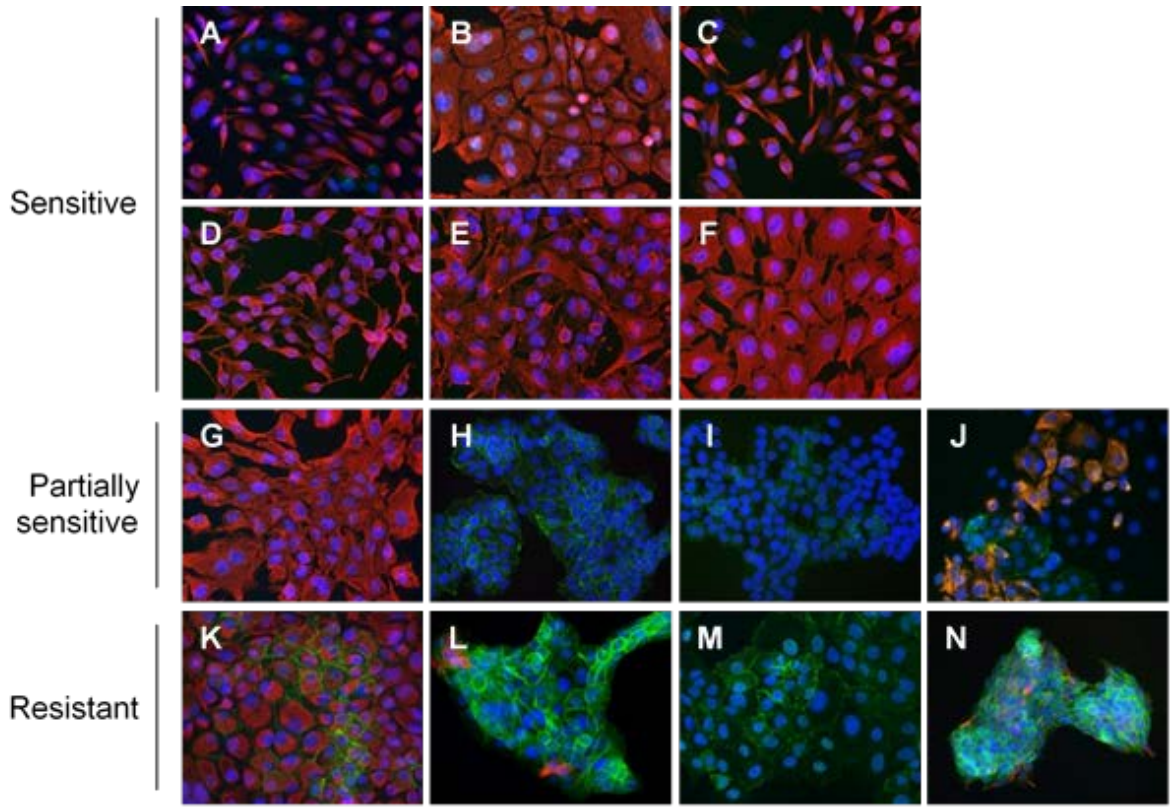


Figure 16. Vimentin and E-cadherin expression and subcellular localization

Merged images of the NCI-60 cell lines immunostained for vimentin (red, cytoplasmic), E-cadherin (green, membrane, cytoplasmic or nuclear), and Hoechst 33342 (blue, nucleus). A: PC-3, B: HOP-92, C: SK-MEL-5, D: MDA-MB-435, E: SF-295, F: SF-539, G: HS-578T, H: T47D, I: HCT-116, J: IGROV1, K: DU-145, L: NCI-H322M, M: OVCAR3, and N: KM-12 cell lines. Cell lines are labeled as atorvastatin-sensitive (A-F), -partially sensitive (G-J) or -resistant (K-N) based on the results of cell proliferation studies shown in Figure 2.

Taken together with Figure 15, the data in Figure 16 suggest that localization but not total expression levels of E-cadherin are significant biomarkers for resistance to atorvastatin. The reduction in cellular cohesion that accompanies EMT depends on decreased E-cadherin functionality, which can occur both transcriptionally and post translationally, the latter specifically relating to protein internalization and subsequent degradation[914]. As such, determination of E-

cadherin localization provides more information regarding functionality (epithelial versus mesenchymal phenotype) than total expression levels.

5.3.5 Forced expression of cell surface E-cadherin converts atorvastatin-sensitive cells to a more atorvastatin resistant state

Given the observed correlation between atorvastatin-resistance and cell membrane E-cadherin expression, we aimed to determine whether exogenously expressed membrane E-cadherin would induce a resistant state in a statin-sensitive cell line. To this end, we used derivatives of a highly statin-sensitive MDA-MB-231 cell line, which express either dsRED (RFP) or dsRED and cell surface E-cadherin (Ecad)[45]. The RFP and Ecad cell lines were treated with log-dilutions of atorvastatin and their cell numbers were determined 3 days after treatment by crystal violet staining. We found that the half maximal inhibitory concentration of atorvastatin (IC_{50}) in MDA-MB-231 cells shifted from 1.16μM to 4.3μM when cell surface E-cadherin was expressed (Figure 17A). A representative image of RFP and Ecad cells treated with 3μM atorvastatin illustrates this shift by showing a relatively higher cell density in the latter (Figure 17B,C). Additionally, the more statin sensitive MDA-MB-231 RFP cells appear more rounded or spindle-like after treatment with 3μM atorvastatin (Figure 17B) whereas the MDA-MB-231 Ecad cells demonstrate a more cohesive and epithelial phenotype, with a lower proportion of cells exhibiting cytoskeletal alterations (Figure 17C). These data suggest that forced cell surface E-cadherin expression can confer partial statin resistance to formerly statin-sensitive cancer cells.

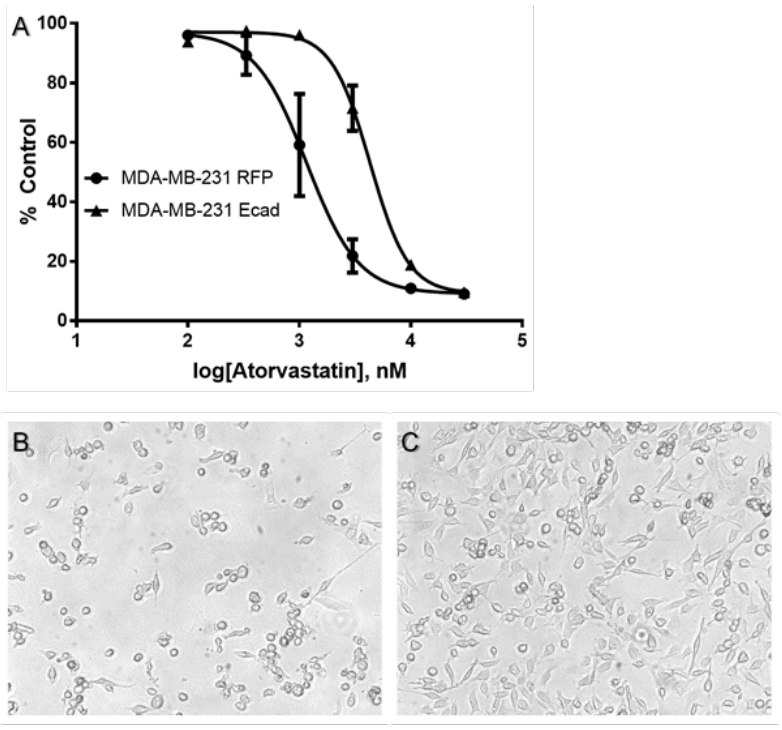


Figure 17. Forced cell surface E-cadherin expression increases cellular resistance to atorvastatin

(A) MDA-MB-231 RFP (●) or MDA-MB-231 RFP E-cadherin (▲) cells were treated with 0.1µM, 0.3µM, 1µM, 3µM, 10µM, or 30µM atorvastatin. Cell viability was assessed by crystal violet staining 3 days after atorvastatin treatment and normalized to the 0.3% DMSO control. Error bars represent the standard deviation (n=3). The data were fit with a sigmoid function to extrapolate the IC50 value (lines). (B) MDA-MB-231 RFP and (C) MDA-MB-231 RFP E-cadherin cells were treated with 3µM atorvastatin. Imaging was done with 40X oil objective lens 3 days after the treatment.

That the localization of E-cadherin to the membrane is critical to induction of resistance to statin therapy may be related to several factors. Previous studies have demonstrated that E-cadherin homotypic complexes result in survival signaling through the Akt-PBK and MAPK pathways[914–916]. These signals that result from cellular cohesion may decrease the potency of the growth suppressive effects of atorvastatin. Our lab has previously demonstrated re-expression of E-cadherin upon metastatic seeding of breast and prostate tumor cells[45,748,917]. These data that

demonstrate exogenously expressed E-cadherin on the membrane increases the resistance of cells to atorvastatin suggest that epithelioid micrometastases present at the metastatic site following MErT would be less susceptible to atorvastatin therapy. In contrast, cells that undergo a secondary EMT to emerge as dedifferentiated macrometastases would regain sensitivity to statin therapy.

5.4 SUPPLEMENTAL DATA

In order to further investigate the mechanism of atorvastatin-mediated growth suppression of tumor cells, we investigated whether supplementation with mevalonic acid - the product of the HMGCR-catalyzed reaction (Figure 11) – would reverse these effects. We found that mevalonate reversed the atorvastatin-induced growth inhibition of the ten cancer cell lines that displayed full or partial sensitivity to atorvastatin (Figure 13, Figure 18).

The data in Figure 18 demonstrate that mevalonic acid supplementation is able to reverse the phenotypic changes induced by atorvastatin in both fully and partially sensitive statin cell lines. This suggests that the mechanism of action of statins in inhibiting tumor cell growth is by direct inhibition of HMGCR. Furthermore, we demonstrate that the rescue of these statin-mediated effects is dose dependent (Figure 19). These data indicate that the growth suppressive effects of atorvastatin are due to the depletion of mevalonate in tumor cells by the suppression of its synthesis by HMGCR.

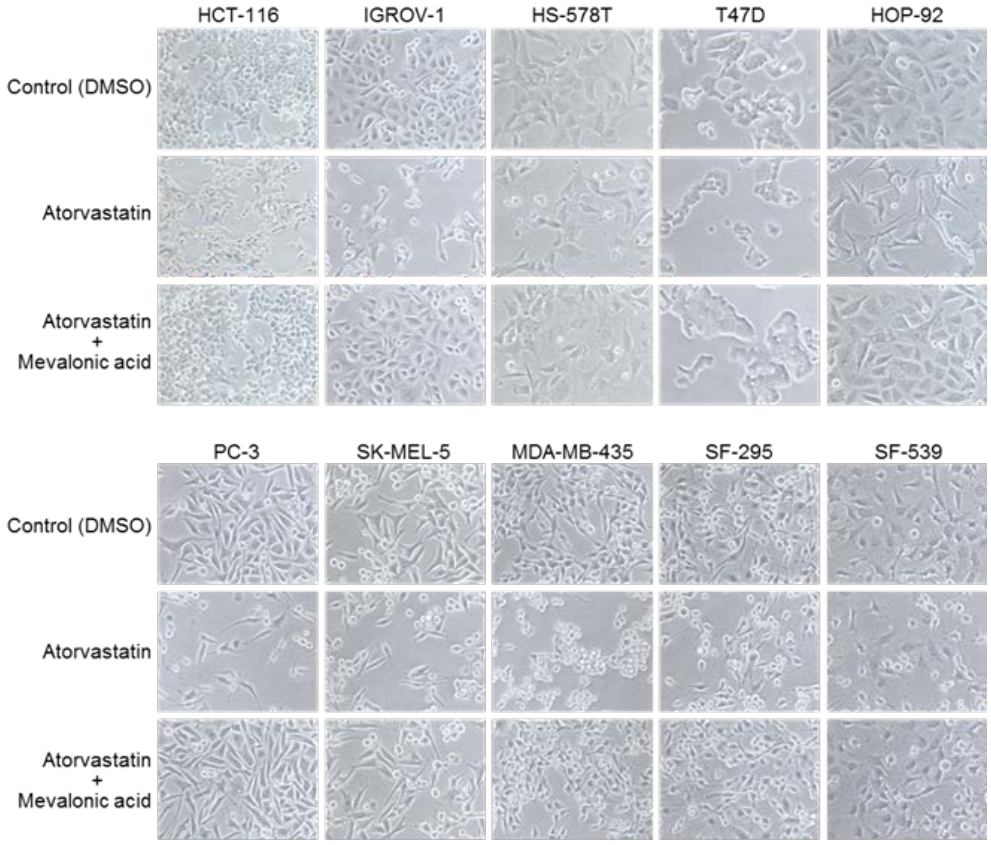


Figure 18. Exogenous mevalonate prevents atorvastatin-induced growth inhibition

Fully- and partially statin sensitive cancer cell lines were treated with 0.1% DMSO (top rows), with 10μM atorvastatin (middle rows) or with 10μM atorvastatin and 100μM mevalonic acid for 24 hours. The microphotographs of the cell cultures are shown demonstrating that mevalonic acid abrogates the atorvastatin-induced growth inhibition.

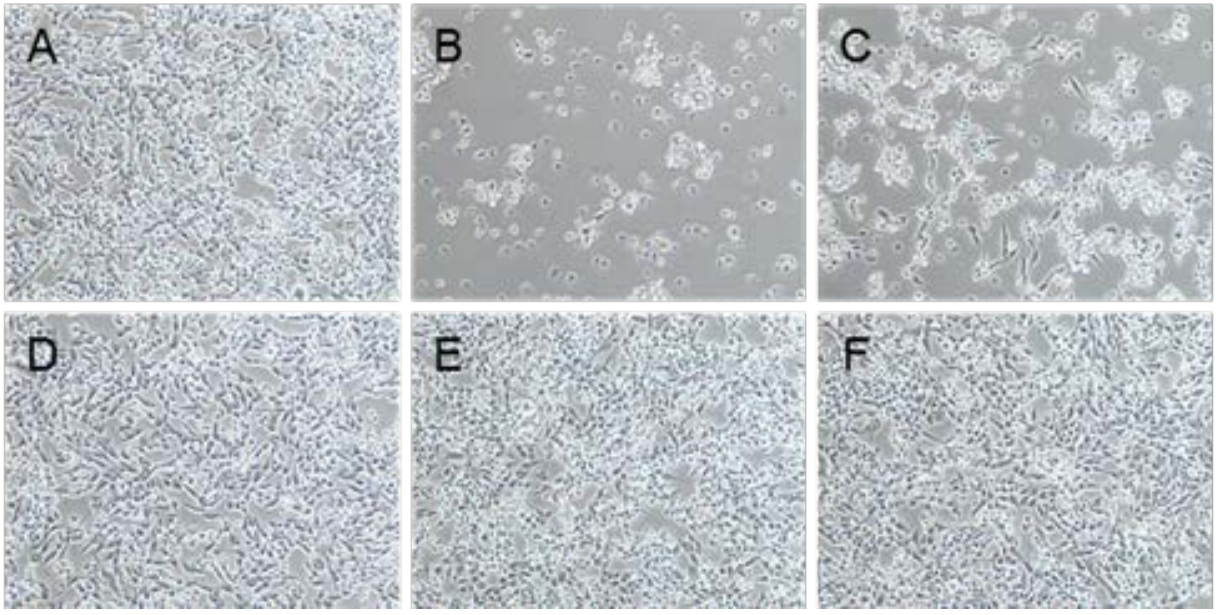


Figure 19. Mevalonate bypasses atorvastatin inhibition in a dose-dependent fashion

The statin sensitive cancer cell line, MDA-MB-435, was treated with 10μM atorvastatin and increasing concentrations of mevalonic acid for 72 hours. (A) Control (DMSO), (B) 10μM atorvastatin, (C) 10μM atorvastatin and 25μM mevalonic acid, (D) 10μM atorvastatin and 50μM mevalonic acid, (E) 10μM atorvastatin and 100μM mevalonic acid, (F) 10μM atorvastatin and 200μM mevalonic acid. The microphotographs of the cell cultures are shown demonstrating that mevalonic acid abrogates atorvastatin-induced growth inhibition in a concentration dependent manner.

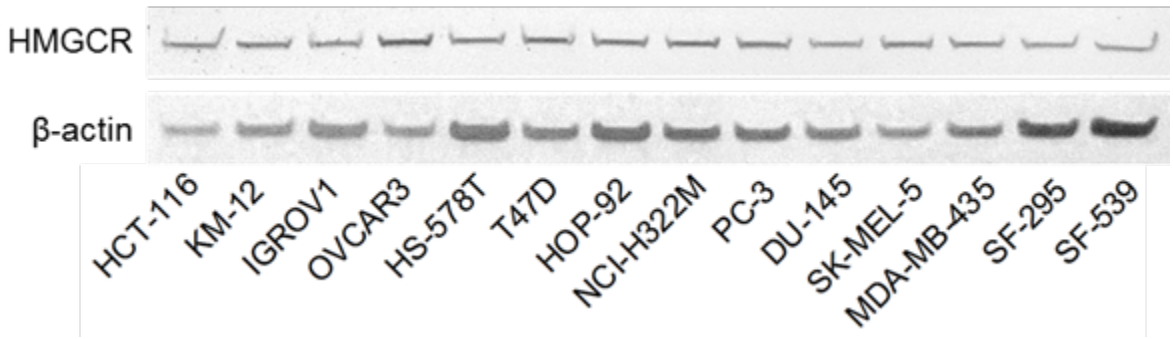


Figure 20. HMGCR expression of NCI-60 cancer cell lines

The expression levels of HMGCR in the indicated fourteen NCI-60 cell lines are shown on western blot together with control β-actin expression.

In order to determine whether atorvastatin sensitivity was dependent on the abundance of the target enzyme HMGCR, we probed the total protein levels by western blot. We found that all fourteen cell lines express HMGCR at comparable levels (Figure 20), demonstrating that statin sensitivity is not due to differential enzyme expression.

To further support our conclusions in Figure 14 that suggest the degree of sensitivity to statin therapy is denoted by post-atorvastatin treatment levels of cholesterol, we conducted Fillipin staining in the remaining 11 cell lines not shown in Figure 14 and present representative images for all 14 cell lines (Figure 21). These cell lines show identical behavior to those presented in Figure 14, namely that the atorvastatin sensitive cell lines exhibit the largest reduction in intracellular cholesterol, the partially sensitive cell lines show a partial reduction, and the resistant cell lines show no reduction.

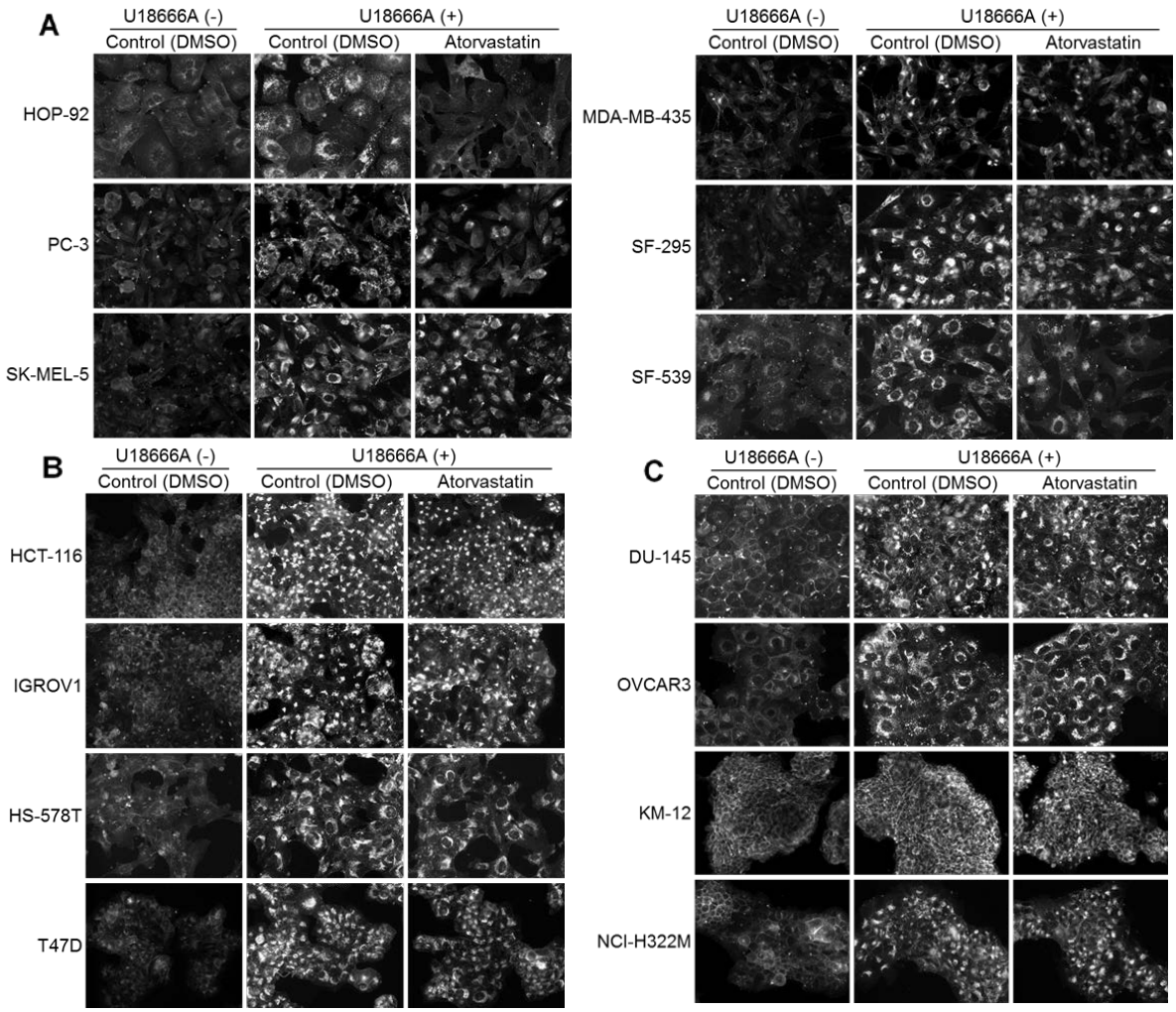


Figure 21. Cholesterol content of atorvastatin treated NCI-60 cancer cell lines

(A) atorvastatin-sensitive cell lines, (B) partially sensitive cell lines, and (C) atorvastatin-resistant cell lines. The cells were treated with 10μM atorvastatin and 1.25μM U18666A for 24 hours. The cells treated with 0.1% DMSO served as a control. Cholesterol was visualized by Filipin III staining.

To further confirm that atorvastatin reduces the cells' cholesterol level through HMGCR inhibition, we measured the cholesterol content in the two most atorvastatin-sensitive cell lines (HOP-92 and PC-3) 24 hours after the atorvastatin treatment in the presence or absence of mevalonate supplementation. As expected, we found that the addition of mevalonate to the growth medium prevented the atorvastatin-induced depletion of cholesterol typically seen in atorvastatin sensitive cell lines (Figure 22). These data suggest that supplemented mevalonic acid is able to integrate into the mevalonate pathway (Figure 14) and overcome the lack of endogenous mevalonic acid that occurs with statin treatment.

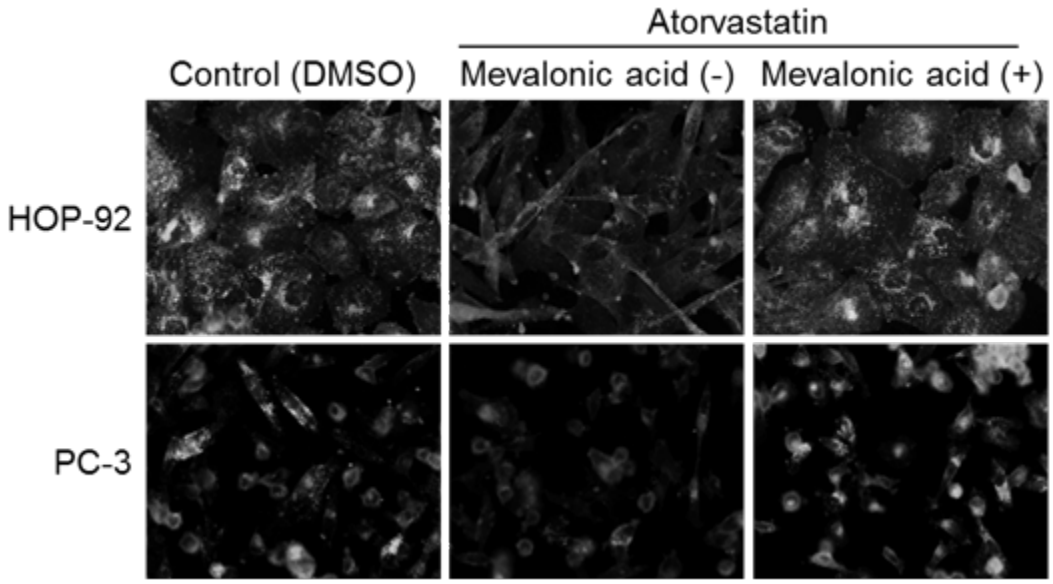


Figure 22. Cholesterol levels of atorvastatin treated cell lines in the presence of mevalonic acid

HOP-92 (top row) and PC-3 cell lines (bottom row) were treated with 10μM atorvastatin in the presence or absence of 100μM mevalonic acid for 24 hours. U18666A was used for a cholesterol intercellular trafficking inhibitor. The cells treated with 0.1% DMSO served as a control. Cholesterol was visualized by Filipin III staining.

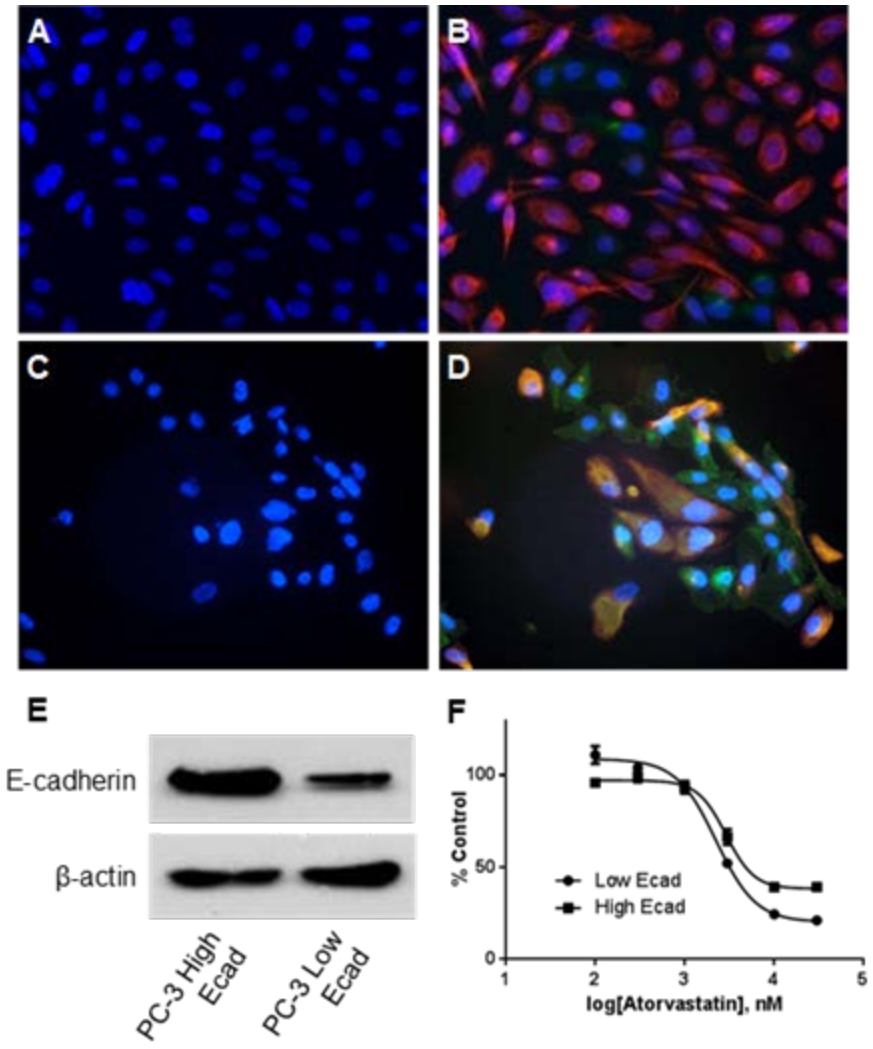


Figure 23. Cytoplasmic expressing E-cadherin PC-3 cells are atorvastatin sensitive

(A, B) PC-3 low E-cadherin and (C, D) PC-3 high E-cadherin cells were stained for E-cadherin (green) and vimentin (red) expression, and counterstained with Hoechst 33342 (blue, nuclear). (E) Total E-cadherin and β-actin determination by western blot. (F) Atorvastatin IC50 curves for the PC-3 low and high E-cadherin cell lines.

To further characterize the role of E-cadherin in mediating statin resistance, we sought to determine whether higher E-cadherin expression that was not membrane localized would induce statin resistance. We found that membrane E-cadherin expression in MDA-MB-231 cells was sufficient to induce resistance to statins (Figure 17). To determine if non-membrane localized E-cadherin could also cause atorvastatin resistance, we employed a PC-3 cell line variant that has higher levels of cytoplasmic E-cadherin expression (PC-3 High Ecad, Figure 23A-E). In contrast to the MDA-MB-231 Ecad cells, PC-3 High Ecad cells did not demonstrate an increased resistance to atorvastatin (Figure 23F).

5.5 SUPPORTING DATA

The following data are unpublished data that were collected in order to further confirm the findings of the previously discussed data that are published. While atorvastatin resistant cell lines have similar protein levels of HMGCR as atorvastatin sensitive cell lines (Figure 20), it is unknown whether atorvastatin treatment influences HMGCR protein levels. To determine whether resistant cell lines can compensate for statin treatment by upregulating HMGCR, statin resistant DU-145 cells were treated with increasing doses of atorvastatin for 24 or 48 hours. We found that statin treatment at both of these time points did not change the protein levels of HMGCR, suggesting that upregulation of this enzyme is not a mechanism of resistance (Figure 24).

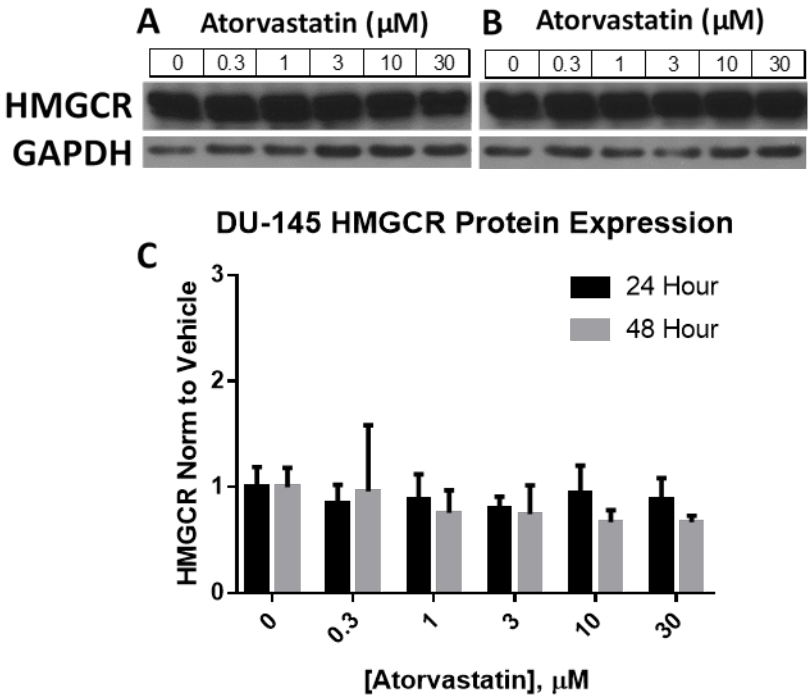


Figure 24. HMGCR expression does not change upon atorvastatin treatment

HMGCR expression in DU-145 after (A) 24 hours or (B) 48 hours of atorvastatin treatment at the indicated concentrations. Total HMGCR protein was detected by western blot. (C) HMGCR bands were quantified using densitometry and normalized to GADPH and vehicle at both time points.

We demonstrated that mevalonic acid supplementation was able to rescue atorvastatin-mediated growth suppression in tumor cells and restore pre-treatment levels of intracellular cholesterol. To determine whether cholesterol supplementation would have the same rescue effect, statin sensitive PC-3 cells were treated with increasing doses of atorvastatin and supplemented with 1μM, 5μM, or 10μM cholesterol. In contrast to mevalonic acid, cholesterol supplementation was not able to rescue atorvastatin-mediated growth inhibition of PC-3 cells (Figure 25). If this were the case, a right-dose shift would be expected as was seen with E-cadherin membrane expression in MDA-MB-231 (Figure 17).

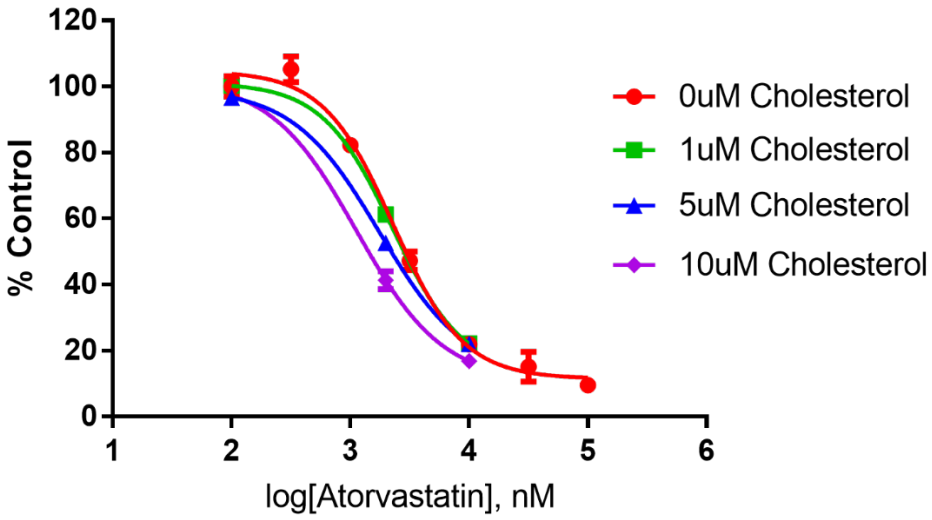


Figure 25. Cholesterol supplementation does not rescue atorvastatin efficacy

PC-3 cells were treated with atorvastatin for 72 hours and supplemented with 1µM, 5µM, or 10µM cholesterol. Cell number was determined by crystal violet staining.

To determine whether the inability of cholesterol to rescue atorvastatin treatment is due to its inability to be used by the cells, the cholesterol uptake was determined by using a green fluorescent cholesterol and quantifying fluorescent signal per cell. In all cell lines tested, atorvastatin decreased cholesterol uptake, except PC-3 which had very low basal levels of cholesterol uptake compared to the other cell lines (Figure 26). A cholesterol trafficking inhibitor U-18666A was able to increase cholesterol uptake in atorvastatin treated cells. Interestingly, these data are the opposite of what is observed in hepatocytes, namely that statin inhibition of HMGCR enhances cholesterol uptake due to upregulation of LDL-R[571]. However, as free cholesterol was used for this assay, it is possible that differential handling of LDL (receptor mediated) and free cholesterol (endocytic) can account for this discrepancy.

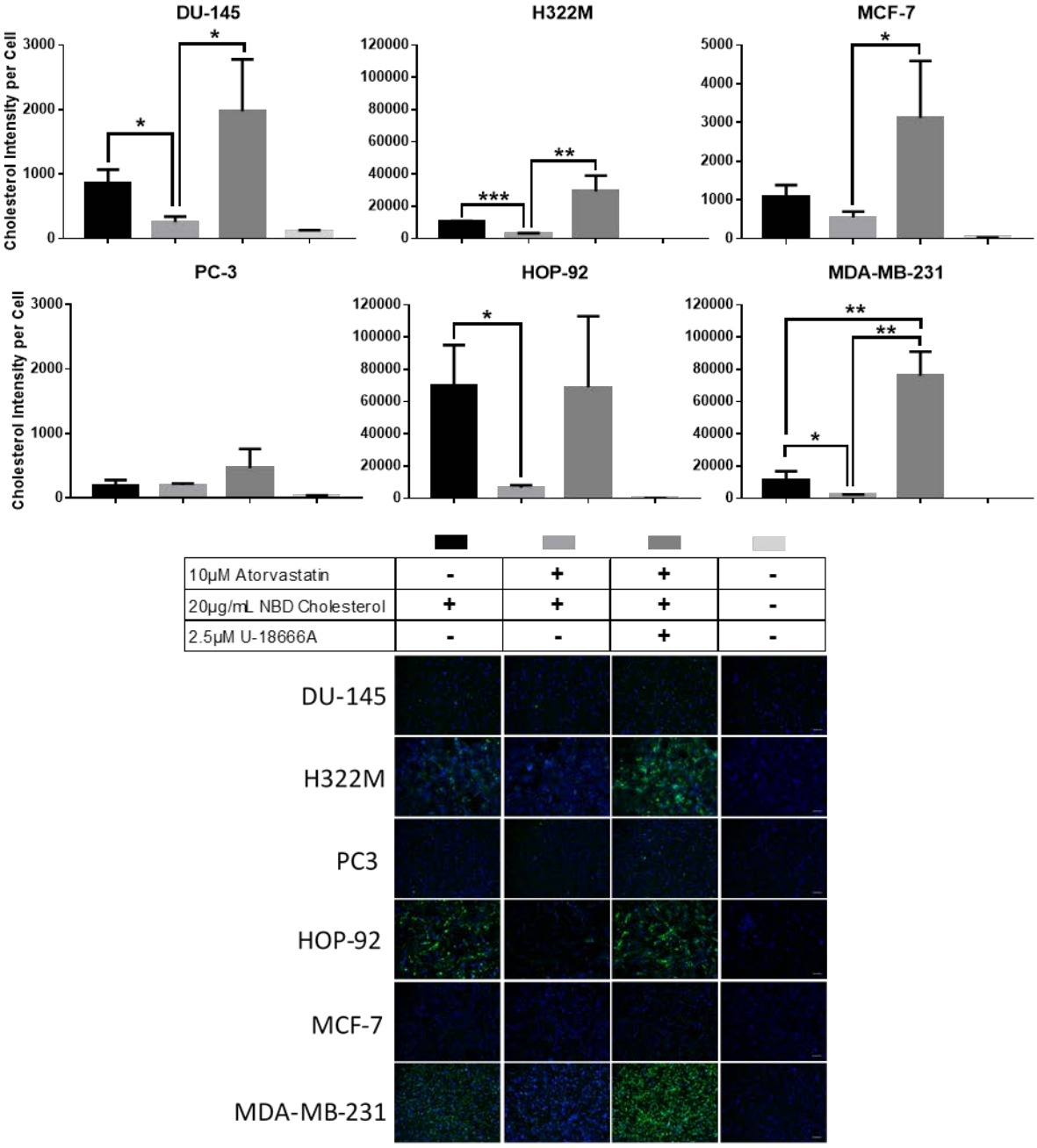


Figure 26. Cholesterol uptake in atorvastatin treated cells

Cells were treated with vehicle (0.02% DMSO) or 10μM atorvastatin, supplemented with 20μg/mL NBD cholesterol for 24 hours. A cholesterol trafficking inhibitor, U-18666A, was used with vehicle at a concentration of 2.5μM as a positive control and vehicle without cholesterol was used as a negative control. Scale bar = 100μm.

The data shown in Figure 25 and Figure 26 suggest that depletion of products of the mevalonate pathway aside from cholesterol may be responsible for atorvastatin's anti-tumor effects. For example, a mevalonate pathway-produced metabolite, geranylgeranyl pyrophosphate inhibits Rho GTPases, which activate transcription factors YAP and TAZ of the Hippo pathway[694,918,919] (Figure 11). Moreover, farnesyl pyrophosphate is known to prenylate Ras, a key regulator of multiple downstream cascades such as PI3K-Akt-PKB and the MAPK pathway[920]. Therefore, statin inhibition of HMGCR may arrest cancer cell proliferation by inactivating downstream signaling proteins and transcription factors.

5.6 CONCLUSIONS

This study demonstrates the potential for statins to be used as anti-tumor agents due to their growth suppressive effects. Importantly, not all tumor cells are sensitive to statin treatment. The cells that exhibit sensitivity to statins show a reduction in intracellular cholesterol levels upon treatment, confirming that statin-mediated shutdown of the mevalonate pathway is critical to the observed effects. This was further confirmed by rescue studies that demonstrate the ability of mevalonate, but not cholesterol, to rescue statin-mediated growth suppression in sensitive cell lines. To demarcate statin sensitive from statin resistant cells, we found that membrane-localized E-cadherin was both a marker and mechanism for statin resistance, confirmed by the inability of increased cytoplasmic E-cadherin to contribute to statin response. In contrast, expression of the target enzyme and total E-cadherin levels were not found to be predictive of statin sensitivity.

The most relevant component of this study to the overarching hypothesis of this project is the influence of E-cadherin on statin susceptibility. Upon metastatic seeding, cells undergo MErT

and re-express E-cadherin on the membrane[45,748,914]. Upon stimulation by the microenvironment, these cells can undergo a secondary EMT to outgrow as mesenchymal de-differentiated masses. As such, statin therapy should be the most effective against the metastatic cells primed to outgrow, as the epithelial micrometastases would be relatively more statin resistant. A selectivity for suppression of emergent cells may explain the clinical reduction in cancer-specific mortality seen in breast cancer patients taking statins.

The next components of this thesis will focus on attempts to better characterize the growth suppressive effects of statins from a mechanistic perspective and identify alternative biomarkers that demarcate statin sensitive and resistant cells. Moreover, statins will be tested in sophisticated *ex vivo* and *in vivo* models of breast cancer metastasis to determine whether statins are indeed able to reduce the proliferation and outgrowth of these eventually mortal micrometastases.

6.0 LIPOPHILIC STATINS LIMIT CANCER CELL GROWTH AND SURVIVAL, VIA INVOLVEMENT OF AKT SIGNALING

6.1 PROLOGUE

The second piece of work contributing to this thesis was published in PloS One in May, 2018[4] . It is one of the two submitted first author publications that have resulted from my thesis work. The majority of this chapter is taken directly from that manuscript, and the corresponding reference can be found both at the beginning of this document and in the references section. Of note, the "Primary Data" section refers to a discussion of the data published in the body of the manuscript, and the "Supplemental Data" section refers to data published in the supplement of the manuscript.

In this work, we investigated the growth suppressive effects of multiple statin drugs on a panel of cancer cell lines. We found that high affinity, lipophilic statins (atorvastatin and simvastatin) suppressed cancer growth with higher efficacy than the hydrophilic statins (rosuvastatin and pravastatin). When comparing atorvastatin to rosuvastatin, we find that the former is more effective at suppressing breast cancer proliferation and can even induce cell death *in vitro*. To examine the cellular response of breast cancer cells to atorvastatin, we determined the prenylation status of Ras, a critical signaling node for both the MAP kinase and PI3K-Akt pathways. We found that statin sensitive breast cancer cells showed a time-dependent decrease in Ras prenylation, which was not seen in statin resistant cells. Downstream of Ras, we found that the PI3K-Akt pathway was the primary pathway suppressed by atorvastatin. Moreover, combined use of a PI3K inhibitor with atorvastatin was able to potentiate atorvastatin efficacy.

These data are a significant foundation to this body of work for several reasons. First, this manuscript demonstrates the role of statin pharmacologic properties in guiding anti-tumor efficacy. Namely, the most effective statins have high binding affinity for the target enzyme, HMGCR, and are lipophilic. Second, we found that atorvastatin-mediated growth suppression is a result of both suppressing proliferation and reducing survival of cancer cells. Finally, these data show that statins suppress signaling through Ras and Akt, which contributes to their anti-tumor effects. As discussed in Chapter 1 above, these proteins play critical roles in breast cancer progression, metastasis, and emergence from dormancy. Since the overarching hypothesis of this thesis involves the ability of statins to target metastatic-emergent breast cancer to reduce recurrence and mortality, these data support this theory by suggesting statins can suppress emergence from dormancy by reducing signaling through Ras and Akt.

6.2 MATERIALS AND METHODS

Cell Sourcing and Cell Culture: All cell lines were sourced directly from the ATCC. Cell lines derived from the following tumor primary sites were employed: breast (MCF-7 and MDA-MB-231), prostate (DU-145), brain (SF-295), and melanoma. DU-L and DU-H describe DU-145 cells, both directly sourced from the ATCC, with low and high levels of E-cadherin respectively, and are described more comprehensively in the following reference[921]. MCF-7 RFP, MDA-MB-231 RFP, MDA-MB-231 RFP/Ecad, and DU-145 RFP were previously developed in our lab by stable transfection of RFP[45]. All cells were maintained in RPMI 1640, GlutaMax Supplement (Gibco, ThermoFisher Scientific) supplemented with 10% HI-FBS (Gemini Bioproducts) and 0.5% penicillin-streptomycin (Gibco, ThermoFisher Scientific), henceforth referred to as RPMI. Stably

transfected RFP or RFP/Ecad cells were maintained with additional supplementation of puromycin (Gibco, ThermoFisher Scientific) or G418 (Teknova) at the following concentrations: 1μg/mL puromycin (MCF-7 RFP), 5μg/mL puromycin (MDA-MB-231 RFP), 900μg/mL G418 (MDA-MB-231 RFP/Ecad and DU-145 RFP). Antibiotic selection media was removed prior to beginning experiments. Primary human hepatocytes were obtained as isolates from excess pathology specimens at UPMC as part of an NIDDK-funded Liver Tissue and Cell Distribution System run by Dr. David Gellar and funded by NIH contract #HHSN276201200017C. Hepatocytes were isolated by collagenase perfusion for subsequent distribution to investigators.

Chemicals: Atorvastatin (PHR-1422), rosuvastatin (SML-1264), pravastatin (P4498), and simvastatin (S6196) were all obtained from Sigma Aldrich, USA. Atorvastatin and rosuvastatin were dissolved in DMSO at a concentration of 50mM. DMSO was used for both statins to maintain carrier consistency between the two primary statins used for this work. Pravastatin was dissolved in sterile milli-Q water at a concentration of 50mM. Simvastatin lactone was dissolved in 200-proof EtOH at a concentration of 50mM. Activated simvastatin was prepared by dissolving simvastatin lactone at a concentration of 50mg/mL in 200-proof EtOH. 1N NaOH was added to a final concentration of 450mN, and the solution was heated in a 50^{o}C water bath for 2 hours. After heating, the solution was stored in aliquots and frozen. Prior to treatment, the pH was lowered to 7.4 by adding 1N HCl. Human EGF (E9644, Sigma, USA) was reconstituted in 1% BSA in normal saline at a concentration of 1mM. PD98059 (S1177, SelleckChem, USA) was reconstituted in DMSO at a concentration of 20mM. LY294002 (S1105 ,SelleckChem, USA) was reconstituted in DMSO at a concentration of 10mM.

Statin IC_{50} Determination: Cells were seeded in 24-well plates at a concentration of $5x10^4$ cells/mL in a volume of 500μL. The next morning, cells were treated with atorvastatin,

rosuvastatin, pravastatin, or simvastatin in half log doses between 100nM and 100μM. The vehicle control treatment used for each statin was 0.2% DMSO for atorvastatin and rosuvastatin, 0.2% EtOH for simvastatin, or complete media for pravastatin. For co-treatment with PD98059 or LY294002, statin solutions were prepared in solutions of the desired PD98059 or LY294002 concentration. After 72 hours of treatment, treatment solutions were aspirated, and cells were fixed with 3.7% formaldehyde (F79-1, ThermoFisher Scientific) for 15 minutes. After fixation, cells were incubated with 0.5% w/v crystal violet for 10 minutes, and excess dye was removed by copious washing with tap water. The absorbed dye was released with 2% SDS, mixed thoroughly before transferring to a 96 well microplate, and read at 560nm using a Tecan SpectraFluor microplate reader (Tecan US, Durham, NC). IC_{50} values were determined by fitting a standard, four-parameter sigmoid curve to the data. All treatments were carried out in triplicate samples, and all data are representative of at least three independent experiments.

Transfection: MDA-MB-231 RFP cells were seeded in 6-well plates at a concentration of $1.5x10^5$ cells/mL in a volume of 2mL. The next morning, cells were transfected in 2mL Optimem (Gibco, ThermoFisher Scientific) with 10μL/well of Lipofectamine RNAi-Max (ThermoFisher Scientific) and either 20nM non-coding (NC) siRNA (Silencer Select Negative Control No. 1 siRNA, Cat # = 4390843, ThermoFisher Scientific) or 20nM HMGCR siRNA (Silencer Select siRNA, siRNA ID = 141, Cat# = 4392420, ThermoFisher Scientific). Four hours after transfection, media was changed to complete media for two hours to allow cells to recover. Cells were then seeded in 24-well plates, as per the "Statin IC_{50} Determination" protocol above, to determine the IC50 of the cells to atorvastatin, pravastatin, and doxorubicin (APP Pharmaceuticals LLC).

Statin Proliferation Assay: MCF-7 RFP, MDA-MB-231 RFP, or MDA-MB-231 RFP/Ecad cells were seeded in 12-well plates on heat-sterilized glass coverslips (Cat# 12-545-80,

ThermoFisher Scientific) at a concentration of $1.5x10^5$ cells/mL in a volume of 1mL. The next morning, cells were treated with atorvastatin or rosuvastatin at doses of 1μM, 5μM, 20μM, or 60μM for 48 hours. Cells treated with 0.12% DMSO served as the vehicle control. After 24 hours of treatment, concentrated EdU (ThermoFisher Scientific) was added to each well to a final concentration of 10μM. After 24 hours of EdU treatment (48 hours of statin treatment), treatment solutions were aspirated, and cells were fixed with 3.7% formaldehyde (F79-1, ThermoFisher Scientific) for 15 minutes and stained for EdU as described below.

EdU Staining: Cells fixed on coverslips were permeabilized with 0.1% Triton-X-100 (ThermoFisher Scientific) for 20 minutes and then washed once with 3% Bovine Serum Albumin (Sigma). Cells were stained for EdU using the Click-iT EdU Alexa Fluor 488 Imaging Kit (ThermoFisher Scientific), per the manufacturer's instructions. After EdU staining, the cells were counter-stained with 2.5μg/mL DAPI for 15 minutes, washed three times in normal saline, and mounted using a glycerol and PVA based mounting medium, courtesy of the Center for Biological Imaging at the University of Pittsburgh. Coverslips were allowed to harden overnight prior to imaging.

Statin Survival Assay: MCF-7 or MDA-MB-231 cells were seeded in 12-well plates at a concentration of $1.5x10^5$ cells/mL in a volume of 1mL. The next morning, cells were treated with 1μg/mL propidium iodide and either complete media (control), 1μM doxorubicin, or 5μM atorvastatin for 72 hours. Cells were imaged on an inverted microscope (Olympus, Model IX70) using a 10x objective to capture both phase contrast and red fluorescence images (561nm) 0, 24, 48, and 72 hours after treatment.

Cytoplasmic and Membrane Protein Extraction: Cells were harvested using 0.25% Trypsin (ThermoFisher Scientific) and quenching with RPMI. Cells were spun at 1000 RPM for 4

minutes and washed once with 1.5mL ice cold PBS with Calcium and Magnesium (Corning). Cells were respun at 1000 RPM for 4 minutes, and the supernatant was carefully aspirated. The cell pellet was resuspended in 50µL 0.2% w/v Saponin (Sigma) supplemented with 1:100 protease inhibitor cocktail V (CalBioChem) and incubated for 15 minutes on ice. After incubation, the membrane was pelleted by centrifuging at 13000g for 15 minutes at 4°C using a refrigerated microcentrifuge (Savant, SFR13K). The supernatant was carefully separated from the membrane pellet and labeled as the cytoplasmic protein fraction. The membrane pellet was washed twice with 1.5mL of 0.2% saponin and the pellet was centrifuged after each wash by spinning at 13000g for 15 minutes at 4°C. Extra washing steps of the membrane were performed to further purify the membrane tethered protein that remained after the initial saponin extraction of the cytoplasm. After the second wash, the membrane protein was eluted by adding ice cold 50µL RIPA buffer (50mM Tris-HCl, 150mM NaCl, 1mM EDTA, 0.1% SDS, 0.5% deoxycholate, 1% NP-40, pH 8.0) supplemented with 1:100 protease inhibitor cocktail V and incubating for 15 minutes on ice. After incubation, the samples were sonicated for 2 seconds (BioLogics Inc., Model 150 V/T) and centrifuged at 13000g for 10 minutes at 4°C. The supernatant was carefully separated from the pellet and labeled as the membrane protein fraction.

Phospho-Protein Extraction: Cells were lysed using RIPA buffer supplemented with 1:100 protease inhibitor cocktail V (CalBioChem, USA) and 1mM Na_3VO_4 and collected into Eppendorf tubes using a cell scraper. The samples were sonicated for 2 seconds and centrifuged at 13000g for 10 minutes at 4°C. The supernatant was carefully separated from the pellet into a new tube for sample preparation.

Active Ras Analysis: The Ras Activation Assay Kit (Cat# 17-218, Millipore Sigma, USA) was used to pull down Ras-GTP with agarose beads GST-tagged with the Raf-1 Ras binding

domain (Raf-1 RBD), per the manufacturer's instructions. MDA-MB-231 RFP cells were treated with or without 1μM atorvastatin for 48 hours, and then cells were stimulated with 5nM EGF for 5 minutes. Cells were lysed using the kit-provided lysis buffer, supplemented with 1:100 protease inhibitor cocktail V (CalBioChem, USA); the lysates were collected into Eppendorf tubes using a cell scraper. The samples were sonicated for 2 seconds and centrifuged at 13000g for 10 minutes at 4^{o}C. The supernatant was carefully separated from the pellet into a new tube for sample preparation. The supernatants were incubated with Raf-1 RBD agarose beads for 45 minutes at 4^{o}C with continuous gentle mixing. After incubation, beads were pelleted at 14000g for 10 seconds and washed three times with lysis buffer. After the third washing step, beads were resuspended in loading buffer, boiled for 5 minutes, and processed per the "Western Blotting" protocol below, taking care to centrifuge agarose beads prior to sample loading. The 'active' form runs at a mobility slightly faster than the inactive form, which is also pulled down, though less efficiently.

Western Blotting: Protein concentration was determined by the BCA Protein Assay Kit (Pierce, ThermoFisher Scientific). Samples were prepared and boiled for 5 minutes prior to loading. Proteins were separated on 9% or 15% Tris Bis-acrylamide gels prepared the same day at 96V (E-C Apparatus Corp., EC-105) until adequate separation was achieved. Samples were transferred onto nitrocellulose membrane at room temperature at 300mA for 1.5 hours (E-C Apparatus Corp., EC135-90). After transferring, membranes were blocked in 5% w/v non-fat dry milk in tris buffered saline with 0.5% Tween-20 (TBS-T). Membranes were probed with primary antibodies in 5% w/v non-fat dry milk overnight at 4^{o}C on a rotator. The primary antibodies used were anti-Pan Ras (1:1000, MA1-012X, ThermoFisher Scientific), anti-MHC-I (1:500, sc-55582, Santa Cruz Biotech), anti-GAPDH (1:20000, G9545, Sigma), anti-E-cadherin (1:1000, 3195S, Cell Signaling), anti-HMGCR (1:1000, ab174830, Abcam), anti-Pan Akt (1:1000, 4691S, Cell

Signaling), anti-Erk1/2 (1:2000, 4695S, Cell Signaling), anti-Phospho-Akt (1:1000, 4060S, Cell Signaling), and anti-phospho-Erk1/2 (1:2000, 4370S, Cell Signaling). After primary incubation, membranes were washed 3 times for 10 minutes each in TBS-T. Species-specific horseradish-peroxidase conjugated secondary antibodies were applied at room temperature in 5% w/v non-fat dry milk for 1 hour. The two secondary antibodies used were anti-rabbit IgG (1:5000, Sigma, A9169) and anti-mouse IgG (1:5000, Sigma, A4416). After secondary incubation, membranes were washed 3 times for 10 minutes each in TBS-T. Membranes were incubated with ECL western blotting substrate (Pierce, ThermoFisher Scientific) and photo developed. Western blots were scanned at a resolution of 300 DPI and grayscale bit depth of 16. Bands were quantified using Image J software.

Immunofluorescence Microscopy: Single field fluorescent images were taken using an Olympus BX40 upright microscope with a 10x objective and fluorescence excitation wavelengths of 405 (DAPI), 488 (Click-iT EdU), and 561 (RFP). Exposures were kept identical across coverslips for each individual experiment for consistency. Image analysis was performed in NIS Elements version 4.5. Nuclei (DAPI), and proliferating nuclei (EdU) channels were labeled using spot detection for bright, clustered spots. The same parameters were used for all images. Once individual channel masks were created, combined channel masks were generated by using the "having" command, which creates a new mask that illustrates all pixels of the first mask that contain at least one pixel of the second. This strategy was used to create a mask for DAPI + EdU (proliferating cell number). After generating all masks, data were measured and extracted for organization and presentation.

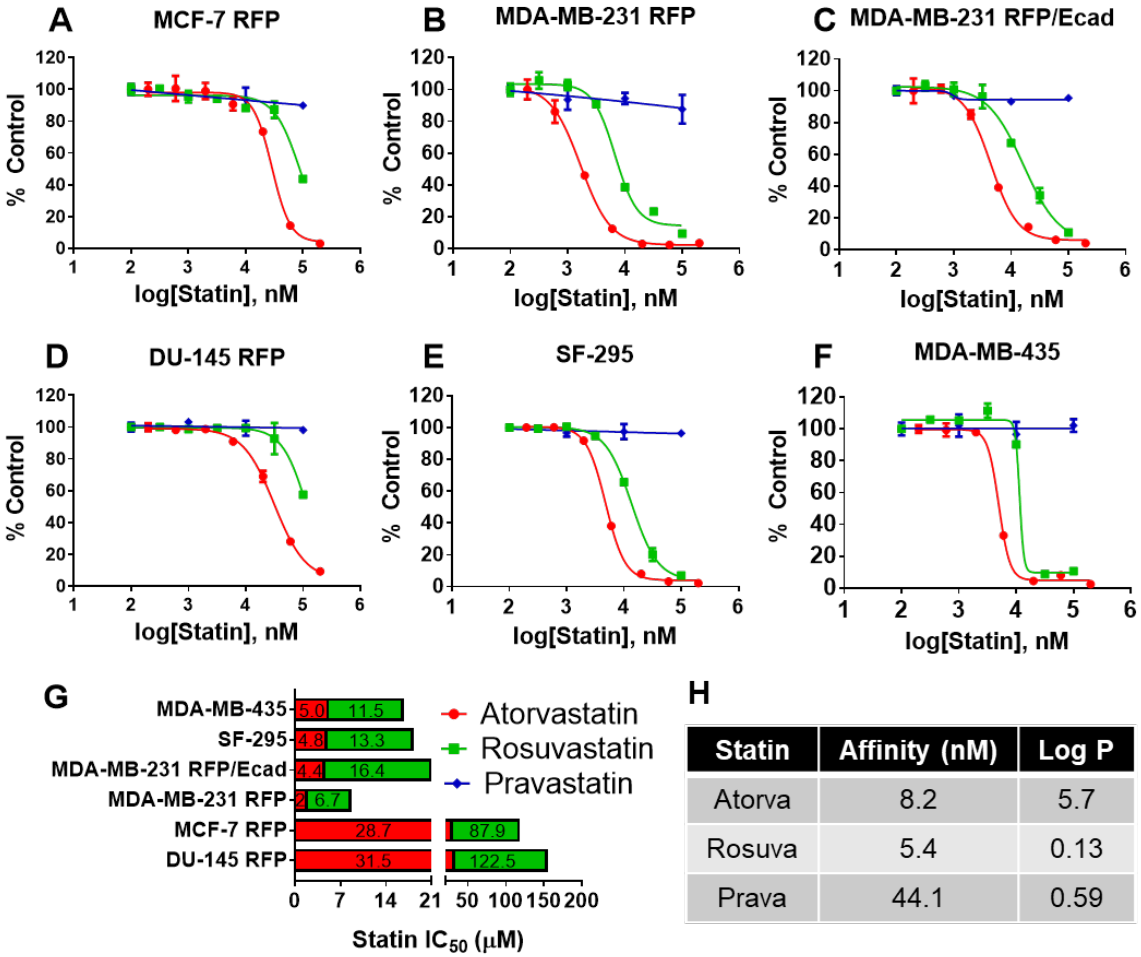
Statistics: Statistics were conducted using GraphPad Prism 7 (Graphpad, USA). In all figures, the data are presented as the mean of three independent experiments conducted in triplicate

with the error bars representing the standard error of the mean. Comparisons of individual columns in Figure 28, Figure 34, and Figure 36 were determined by use of a student's two-tailed unequal variance t-test. Significance levels are reported in the figure legends and are kept consistent across all figures with symbols denoting * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

6.3 PRIMARY DATA

6.3.1 Statins suppress cancer cell growth differentially

We previously demonstrated that atorvastatin could suppress the growth of many cancer cell lines[2]. We were curious whether other statins would show similar growth suppressive effects or whether growth suppression would be governed by the pharmacological properties of the specific statin. To compare to the lipophilic atorvastatin, we also tested rosuvastatin and pravastatin, both hydrophilic statins. While rosuvastatin shares a similar affinity for HMGCR as atorvastatin, pravastatin has approximately a 10-fold lower affinity for the enzyme (Figure 27H).



Statin	Affinity (nM)	Log P
Atorva	8.2	5.7
Rosuva	5.4	0.13
Prava	44.1	0.59

Figure 27. Lipophilic statins are more effective than hydrophilic statins

Lipophilic atorvastatin is more effective at suppressing cancer cell growth than hydrophilic rosuvastatin or pravastatin. Dose response curves for (A) MCF-7 RFP, (B) MDA-MB-231 RFP, (C) MDA-MB-231 RFP/Ecad, (D) DU-145 RFP, (E) SF-295, and (F) MDA-MB-435 cancer cells when cultured with atorvastatin (red), rosuvastatin (green), or pravastatin (blue) for 72 hours. Cell number was determined by crystal violet staining. (G) Sigmoidal curves were fit to the dose response data, and IC50 values of atorvastatin and rosuvastatin in each cell line were extrapolated. (H) Pharmacologic parameters of atorvastatin, rosuvastatin, and pravastatin as found in the literature[562,922]. All data are representative of at least three independent experiments.

We found that atorvastatin was the most effective and pravastatin was the least effective at suppressing the growth of cancer cell lines, including breast (MCF-7 RFP, MDA-MB-231 RFP, and MDA-MB-231 RFP/Ecad, Figure 27A-C), prostate (DU-145, Figure 27D), brain (SF-295, Figure 27E), and melanoma (MDA-MB-435, Figure 27F). Rosuvastatin was 2.3- to 3.9-fold less potent than atorvastatin, even though the former has higher affinity for HMGCR (5.4nM vs. 8.2nM)[562]. Additionally, pravastatin demonstrated no growth suppressive efficacy up to concentrations of 100μM, even in sensitive cell lines. These results suggest that lipophilic, high potency statins are the most effective at suppressing tumor cell growth.

The inefficacy of pravastatin has been previously reported[923] and is likely due to a combination of low affinity for HMGCR and hydrophilicity. Whereas lipophilic statins easily diffuse across the membrane, hydrophilic statins rely more on active transport. The four major statin transporters in the liver are SLCO1B1, 1B3, 1A2, 2B1[592]. While statin transport is handled by all of these carriers, the major part of atorvastatin handling is accomplished by SLCO1B1, which is why we focused on this transporter. Additionally, mutations in the SLCO1B1 gene are known to predispose to statin-induced myopathy[924]. Indeed, exogenous expression of SLCO1B1 has been found to increase pravastatin uptake[798]. Unfortunately, we were unable to stably express SLCO1B1 transporters in our cells to demonstrate this causality. In sum, our data suggest that the low efficacy of both hydrophilic and low affinity statins is due to decreased HMGCR inhibition. This underscores the importance of the mevalonate pathway for cancer cell growth.

6.3.2 Atorvastatin more potently suppresses proliferation of breast cancer cells *in vitro* than rosuvastatin

Since we observed atorvastatin was more effective at suppressing cell growth than rosuvastatin, we wanted to determine if this growth suppression was due to a decrease in cellular proliferation. Focusing on the breast cancer cell lines, we treated MCF-7 RFP, MDA-MB-231 RFP, or MDA-MB-231 RFP/Ecad with atorvastatin for 48 hours, incorporating EdU (a DNA analog) in the last 24 hours of treatment to quantify proliferation (Figure 28A). We observed a dose dependent decrease in the percentage of proliferating cells exposed to both atorvastatin and rosuvastatin in MCF-7 RFP (Figure 28B), MDA-MB-231 RFP (Figure 28C), and MDA-MB-231 RFP/Ecad (Figure 28D). Concurrent with our growth curve data, we found that atorvastatin was more effective at suppressing cell proliferation than rosuvastatin at the same treatment dosage.

We previously and herein demonstrate a higher resistance of membrane E-cadherin expressing MDA-MB-231 cancer cells to atorvastatin than their E-cadherin negative counterpart. When we examined the percentage of cells that had proliferated after 24 hours in the absence of statin treatment, we observed that the E-cadherin expressing MDA-MB-231 cells were roughly 50% less proliferative than the cells lacking E-cadherin (Figure 28, Figure 29). Moreover, similarly to the growth inhibition data, E-cadherin expressing MDA-MB-231 cells demonstrate less of a reduction in proliferating cells with statin treatment (Figure 29).

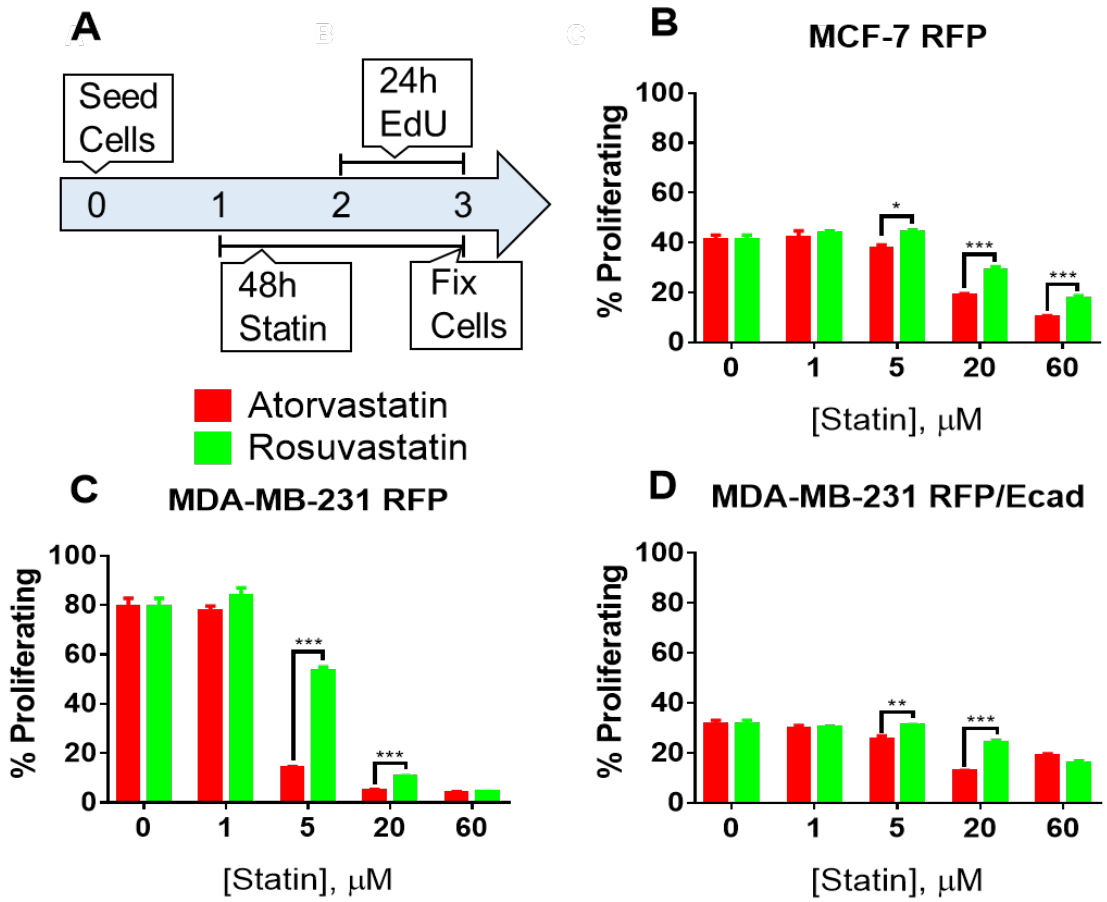


Figure 28. Atorvastatin decreases breast cancer cell proliferation more potently than rosuvastatin

(A) Experimental schematic for assessing the proliferation of breast cancer cells under treatment with atorvastatin or rosuvastatin for 48 hours (B) MCF-7 RFP, (C) MDA-MB-231 RFP, and (D) MDA-MB-231 RFP/Ecad were cultured with atorvastatin or rosuvastatin for 48 hours; during the final 24 hours the media included 10uM EdU. Cells were fixed, EdU was detected, and cells were counterstained with DAPI to label all nuclei. Cellular proliferation was quantified by determining the percentage of EdU positive cells (green, all nuclei are blue - DAPI). All data are representative of at least three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

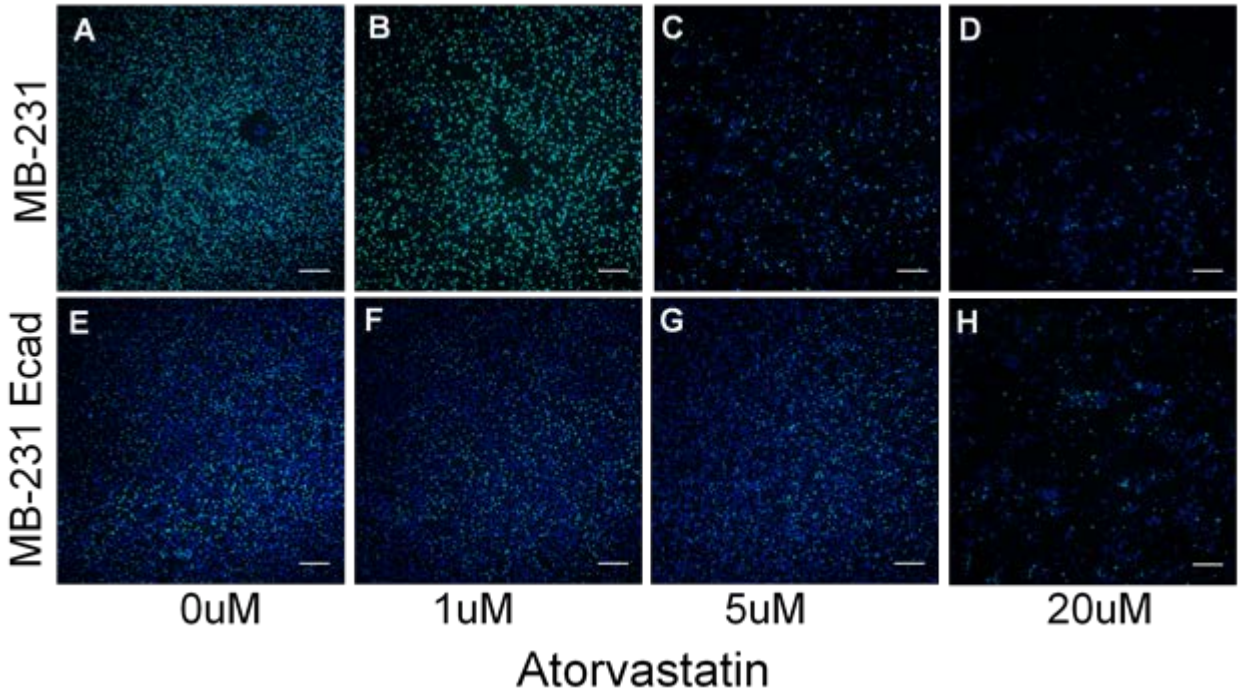


Figure 29. E-cadherin increases resistance to atorvastatin-mediated proliferation suppression

(A-D) MDA-MB-231 RFP and (E-H) MDA-MB-231 RFP/Ecad cells treated with (A,E) 0μM, (B,F) 1μM, (C,G) 5μM, or (D,H) 20μM atorvastatin demonstrate both E-cadherin mediated growth suppression and atorvastatin resistance. Green = EdU, Blue = DAPI. Scale bar = 200μm.

These findings demonstrate that statins exert direct effects on cancer cell proliferation. Other authors have reported direct induction of apoptosis and cell death in tumor cells after statin treatment, in cancer cell lines derived from breast[925], lung[926], and prostate[927] as well as decreases in cell number or viability[537]. In contrast, there are only a few reports of statins reducing proliferation of cancer cells by directly quantifying cycling cells[928]. The majority of studies in the literature use methods that do not distinguish between cell viability and growth, such as MTT and ATP based assays. We show a decrease in proliferating percentage of adherent breast cancer cells

(those that have survived the anti-survival effects of statin therapy), which demonstrates direct effects on cell proliferation (Figure 28).

6.3.3 Atorvastatin treatment induces cellular death in statin sensitive MDA-MB-231 cells but not statin resistant MCF-7 cells

Given the anti-proliferative effects we observed of atorvastatin on breast cancer cells, we next wanted to determine whether atorvastatin also impacts individual cell survival. We treated statin sensitive MDA-MB-231 cells and statin resistant MCF-7 cells with 5μM atorvastatin for 72 hours and assayed cell death by including propidium iodide in the culture medium. We took phase contrast and fluorescent images every 24 hours to assess for cellular uptake of propidium iodide.

We found that atorvastatin reduced the survival of MDA-MB-231 cells, starting 24 hours after treatment (Figure 29A-H). In contrast, the survival of MCF-7 cells was unaffected by atorvastatin, even at 72 hours of treatment (Figure 29M-T). The survival of both cell lines was decreased with 1μM of doxorubicin (Figure 29I-L, U-X).

These data suggest that cell viability is reduced with atorvastatin treatment in cell lines that are susceptible to its growth inhibitory (Figure 27) and anti-proliferative (Figure 28) effects. Importantly, these data are an independent finding from the proliferative data shown in Figure 28, as the latter were normalized to the total adherent cell number, which omits dead cells. As such, statins likely suppress tumor cell growth by two main mechanisms: 1) they decrease tumor cell proliferation (Figure 28), and 2) they decrease tumor cell survival (Figure 30).

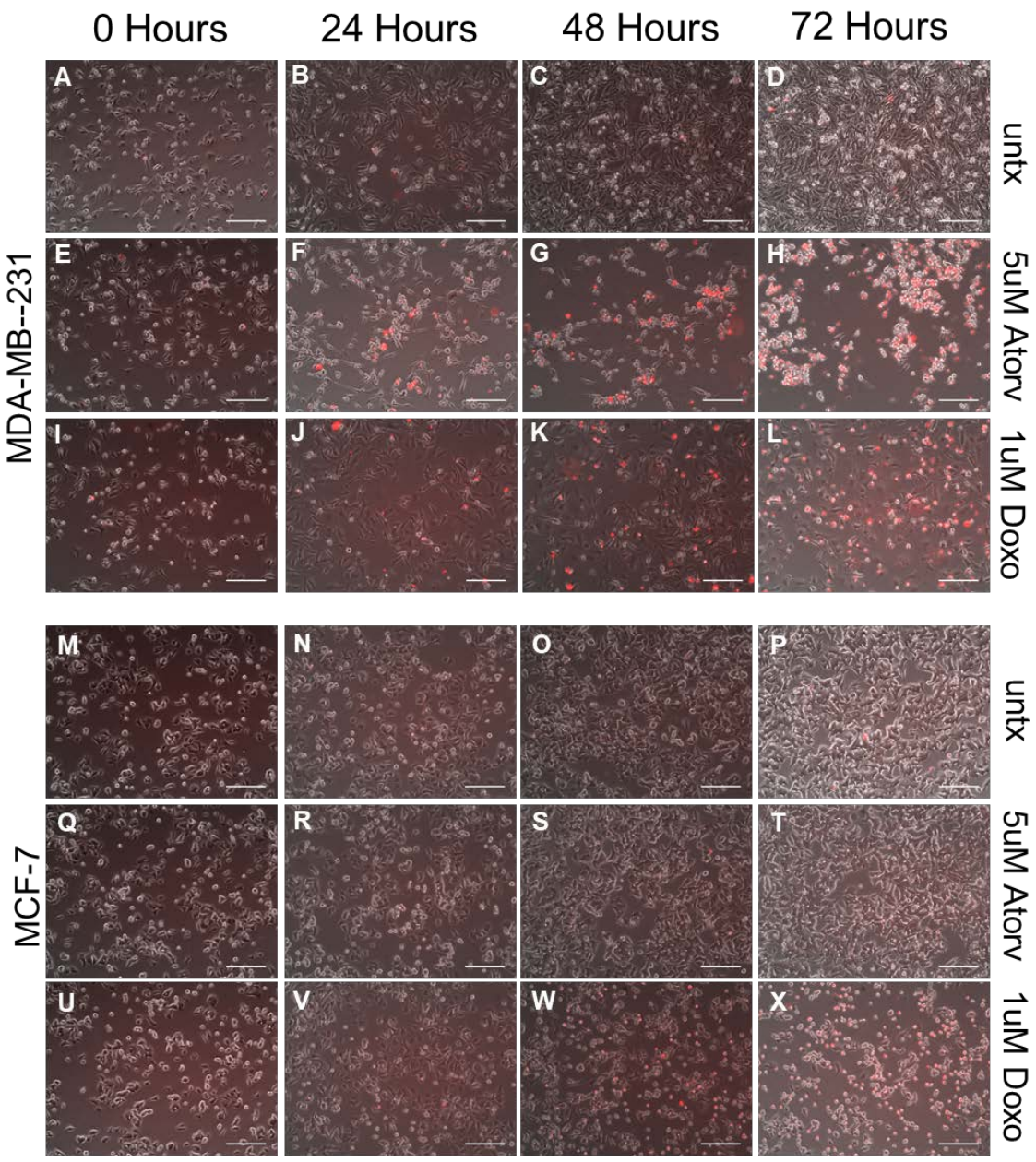


Figure 30. Atorvastatin decreases survival of sensitive but not resistant breast cancer cells

(A-L) MDA-MB-231 or (M-X) MCF-7 breast cancer cells were treated with (A-D and M-P) 0.01% DMSO (untx), (E-H and Q-T) 5μM atorvastatin (5uM Atorv), or (I-L and U-X) 1μM Doxorubicin (1uM Doxo) for (left) 0 hours, (middle-left) 24 hours, (middle-right) 48 hours, or (right) 72 hours in the presence of 1μg/mL propidium iodide. Cells were imaged using an inverted microscope to detect propidium iodide (red) and to look at cell morphology and density using phase contrast microscopy. Scale bar = 200μm. All data are representative of at least three independent experiments.

6.3.4 Atorvastatin treatment decreases the proportion of membrane-tethered Ras in statin sensitive cells

To ascertain the mechanism by which atorvastatin treatment decreases both proliferation and survival, we queried whether a product of HMG-CoA reductase, which can be rescued by mevalonate bypassing the blockade of HMG-CoA reductase, was involved. These products are involved with protein lipidation to redirect otherwise soluble proteins to the cellular membranes. Thus, we investigated effects on the canonical intermediary pathways that contribute to both proliferation and survival in cancer cells, those thru MEK-Erk and PI3-kinase-Akt. Both of these can be activated downstream of Ras signaling, and Ras activation requires juxtamembrane positioning accomplished by protein prenylation.

As such, we investigated Ras prenylation in statin sensitive MDA-MB-231 RFP, and statin resistant MCF-7 RFP cells when treated with 1μM atorvastatin to determine if there is altered Ras signaling or downstream elements. We observe that statin sensitive MDA-MB-231 RFP cells show a 50-fold increase in cytoplasmic Ras and a 50% decrease in membrane-bound Ras over 48 hours of treatment (Figure 31A-C). This is consistent with most of Ras being membrane-associated and statins not completely eliminating the production of geranyl-geranylphosphate. In contrast, Ras localization is unchanged with atorvastatin treatment in statin resistant MCF-7 RFP cells (Figure 32A-C). These data suggest that atorvastatin treatment in statin sensitive cells decreases the proportion of signaling competent Ras.

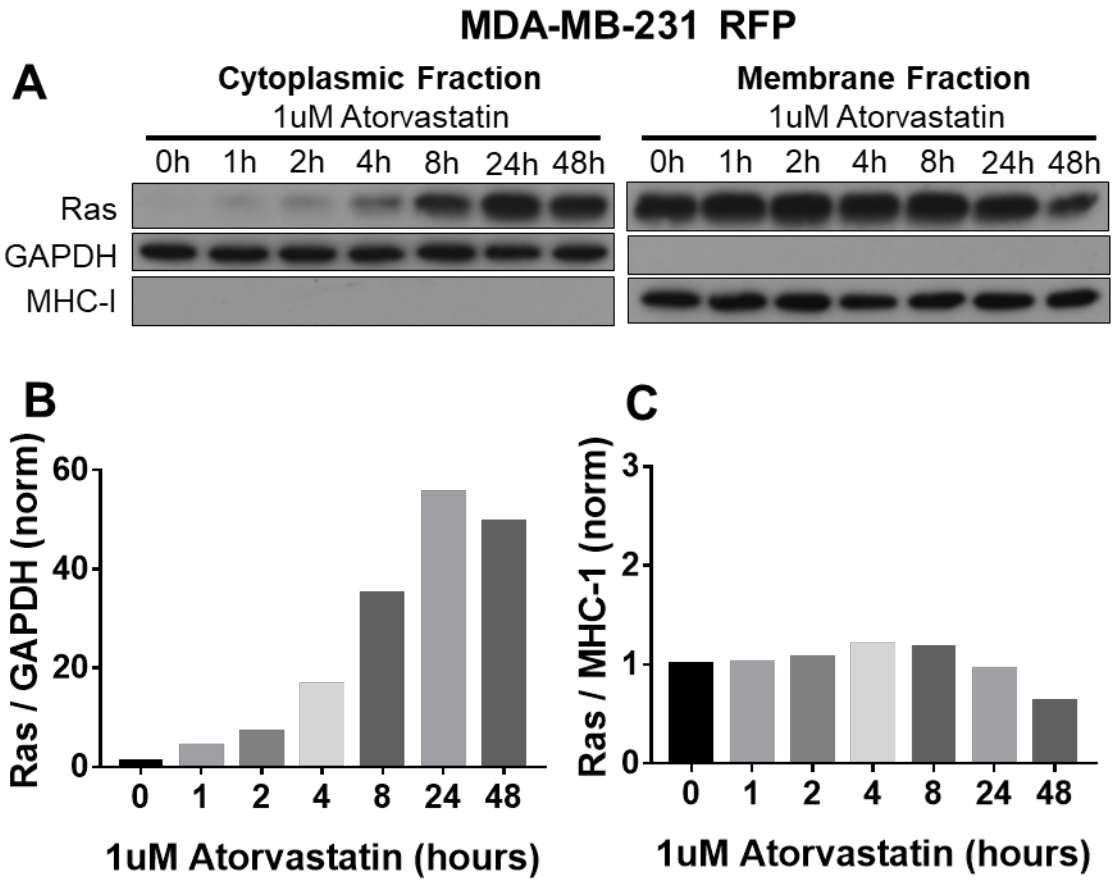


Figure 31. Atorvastatin decreases membrane-bound Ras in statin sensitive MDA-MB-231

MDA-MB-231 RFP cells were treated with atorvastatin for 0, 1, 2, 4, 8, 24, or 48 hours, and protein was collected in cytoplasmic and membrane fractions. (A,B) Cytoplasmic Ras increased in statin treated MDA-MB-231 RFP cells over the course of 48 hours whereas (A,C) membrane Ras decreased. All data are representative of at least three independent experiments.

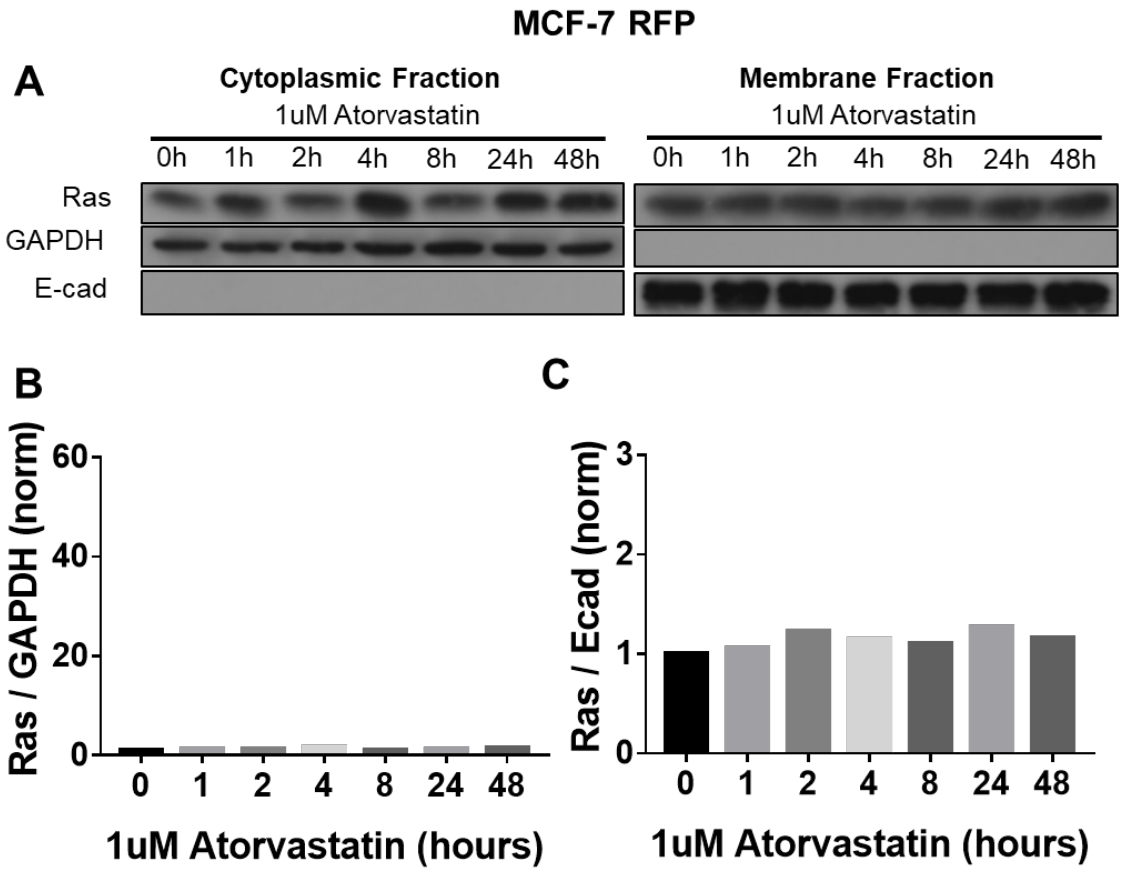


Figure 32. Atorvastatin does not affect membrane-bound Ras in statin resistant MCF-7

MCF-7 RFP cells were treated with atorvastatin for 0, 1, 2, 4, 8, 24, or 48 hours, and protein was collected in cytoplasmic and membrane fractions. (A,B) Cytoplasmic and (A,C) membrane Ras were unchanged by atorvastatin treatment in MCF-7 RFP cells. All data are representative of at least three independent experiments.

It has been postulated that part of the anti-tumor effect of statins is caused by reduction in the prenylation, and thus localization, of signaling proteins such as Ras and the Rho family of proteins[560]. Decreases in membrane anchoring of H-Ras have been shown in breast cancer cells treated with statins[741]. Above, we demonstrate that the localization of Ras is dependent on the statin sensitivity of the cells undergoing treatment. While the statin sensitive MDA-MB-231 RFP cells demonstrate a shift from membrane to cytoplasmic Ras localization over the course of 48 hours of statin treatment, the same increases are not seen in statin resistant MCF-7 RFP cells (Figure 31, Figure 32). Thus, the influence of statins on the prenylation status of Ras, which affects its subcellular localization, correlates with the sensitivity of the cells to statin-mediated growth suppression.

6.3.5 Atorvastatin treatment blunts EGF-stimulated phosphorylation of Erk and Akt in a sensitivity-dependent manner

EGF is a growth factor that signals proliferation and migration in breast cancer and is present as an autocrine stimulatory factor in most all aggressive mammary carcinomas. Our lab has previously shown that EGF stimulation of dormant breast cancer cells can drive outgrowth from dormancy in an *ex vivo* microphysiological system model for breast cancer metastasis to the liver [751,832,898]. Given the importance of EGF in promoting breast cancer growth and metastasis, we wanted to determine whether atorvastatin pre-treatment could influence the phosphorylation of Akt and Erk in breast cancer cells. We pre-treated MCF-7 RFP, MDA-MB-231 RFP, or MDA-MB-231 RFP/Ecad with 5μM atorvastatin for 24 hours. We then stimulated cells with 5nM EGF for either 5 or 30 minutes. We found that atorvastatin pre-treatment was able to blunt EGF-mediated increases in Akt phosphorylation in all three cell lines and Erk-mediated phosphorylation

in MDA-MB-231 RFP/Ecad and MCF-7 RFP cell lines (Fig. 5 A-C). MDA-MB-231 RFP has high levels of tonic Erk phosphorylation due to autocrine EGF signaling[45].

Next, we quantified the blunted EGF response by comparing the vehicle pre-treatment to atorvastatin pre-treatment at each time point for EGF stimulation. With no EGF stimulation, we found that atorvastatin decreased the basal phosphorylation of Akt in statin sensitive MDA-MB-231 RFP cells but not in the more resistant MDA-MB-231 RFP/Ecad or MCF-7 RFP cells. With 5 or 30 minutes of EGF stimulation, we observed that the degree to which atorvastatin pre-treatment decreased EGF-stimulated Akt phosphorylation directly correlated with the sensitivity of these cell lines to atorvastatin-mediated growth inhibition (Figure 27, Figure 33D). These data suggest that the susceptibility to statin-mediated growth inhibition involves inhibition of Akt signaling and are consistent with the decrement in Ras localization to the membrane.

As stated previously, micrometastases often remain in a state of dormancy for years or decades before emerging as clinically evident metastases. Our lab has previously demonstrated that dormant micrometastases can be stimulated to outgrowth in an *ex vivo* microphysiological system model for breast cancer metastasis to the liver by using a combined stimulus of EGF and LPS[832]. To determine whether atorvastatin would influence the EGF responsiveness of our breast cancer cell lines, we treated MCF-7 RFP, MDA-MB-231 RFP, and MDA-MB-231 RFP/Ecad with atorvastatin for 24 hours and then stimulated with 5nM EGF for 5 or 30 minutes. We found that all three cell lines demonstrated increases in Akt and Erk phosphorylation with EGF stimulation and that atorvastatin pretreatment could block these increases in a manner that correlated with their sensitivity to atorvastatin-mediated growth inhibition (Figure 33). The decrease in Akt phosphorylation seen after 30 minutes of EGF treatment is most likely secondary to receptor internalization and degradation due to the high abundance of soluble ligands[929,930].

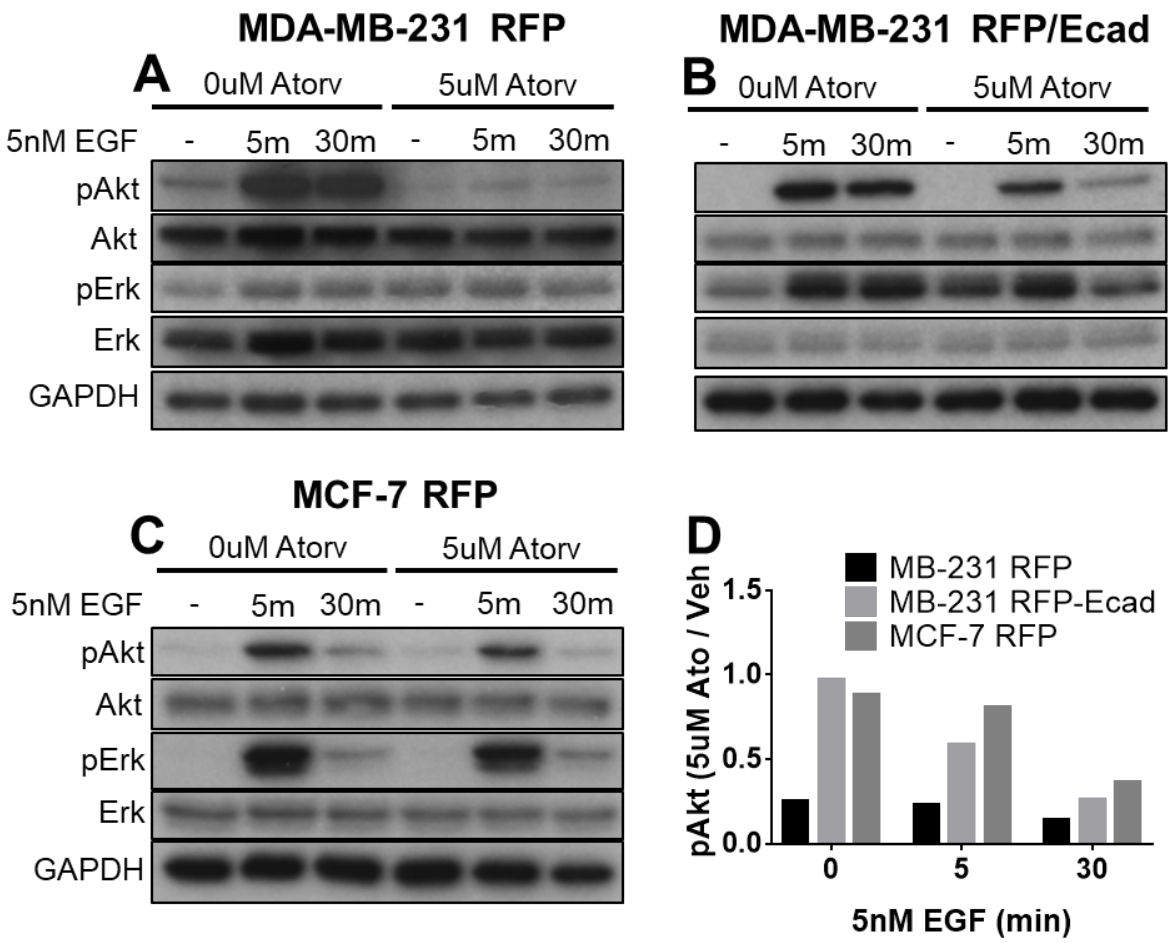


Figure 33. Atorvastatin sensitivity correlates with blunted Akt phosphorylation in response to EGF

(A) MDA-MB-231 RFP, (B) MDA-MB-231 RFP/Ecad, and (C) MCF-7 RFP cells were treated with 5μM atorvastatin for 24 hours and then stimulated with 5nM EGF for 5 or 30 minutes. (D) Akt phosphorylation fold change, defined as the density of pAkt under atorvastatin pretreatment divided by the density of pAkt under vehicle treatment, was quantified for 0, 5, or 30 minutes of 5nM EGF stimulation. All data are representative of at least three independent experiments.

These data suggest that atorvastatin may decrease growth-factor stimulated growth of breast cancer cells. Moreover, the E-cadherin mediated resistance to atorvastatin suppression of Akt stimulation by EGF suggests that epithelial micrometastases that undergo a secondary EMT and outgrow will be more selectively suppressed by statin treatment than those that remain in dormancy. As atorvastatin effects are most noted when E-cadherin is absent from the plasma membrane[2], the cells that are epithelial and dormant as micrometastases would likely be relatively unaffected. However, when these cancer cells undergo a secondary EMT that limits E-cadherin presentation during metastatic outgrowth, they would then be susceptible to statin suppression.

6.3.6 Inhibition of Akt but not Erk signaling is synergistic with atorvastatin-mediated growth suppression

To explore what may potentiate the anti-proliferative effects of atorvastatin, we investigated the two effected pathways: the PI3K-Akt and MAP kinase signaling networks, as both are downstream from Ras signaling and at least partially inhibited by statins. It has previously been shown that both pathways play important roles in breast cancer cell growth and migration[200,237]. To probe these signaling pathways, we employed a pan-PI3K inhibitor (LY294002) and a Mek1/2 inhibitor (PD98059), which inhibit their phosphorylation of Akt and Erk1/2 respectively. As crosstalk between these two pathways has been previously reported, we first determined the effect of each inhibitor on the opposing pathway.

To probe the effects of these two inhibitors on influencing the susceptibility of breast cancer cells to atorvastatin, we co-treated MCF-7 RFP, MDA-MB-231 RFP, or MDA-MB-231 RFP/Ecad with atorvastatin and either PD98059 or LY294002 for 72 hours. With PD98059 and atorvastatin co-treatment, we observed no change in the atorvastatin IC_{50} to cell growth inhibition

(Figure 34A-D). Additionally, we observed an increase in Erk phosphorylation with 5μM atorvastatin treatment in statin sensitive MDA-MB-231 RFP cells at each dose of PD98059 used (Figure 34E,F). In contrast, we observed a dose-dependent potentiation of atorvastatin with LY294002 co-treatment (Figure 34G-J). When probing Akt phosphorylation after atorvastatin treatment, we found that 5μM atorvastatin significantly decreased basal Akt phosphorylation in statin sensitive MDA-MB-231 RFP cells, which was further decreased with LY294002 treatment (Figure 34K,L).

When inhibiting two of the main downstream pathways of Ras, we found that reduction of Akt signaling through PI3K inhibition significantly potentiated the growth suppression of atorvastatin, whereas reduction of Erk signaling through Mek1/2 inhibition did not affect atorvastatin efficacy (Figure 34). Western blotting demonstrated a significant reduction of Akt phosphorylation in atorvastatin treated MDA-MB-231 RFP cells, whereas Erk phosphorylation was increased with atorvastatin treatment (Figure 34). Previous studies have demonstrated crosstalk between the PI3K-Akt and MAPK signaling pathways at the level of Akt and Raf[931]. These findings suggest that the reduction in Akt phosphorylation observed with statin treatment may be the source of the increase in Erk phosphorylation. Others have demonstrated decreases in Akt phosphorylation with statin treatment in prostate[689] and breast[541] cancer cell lines.

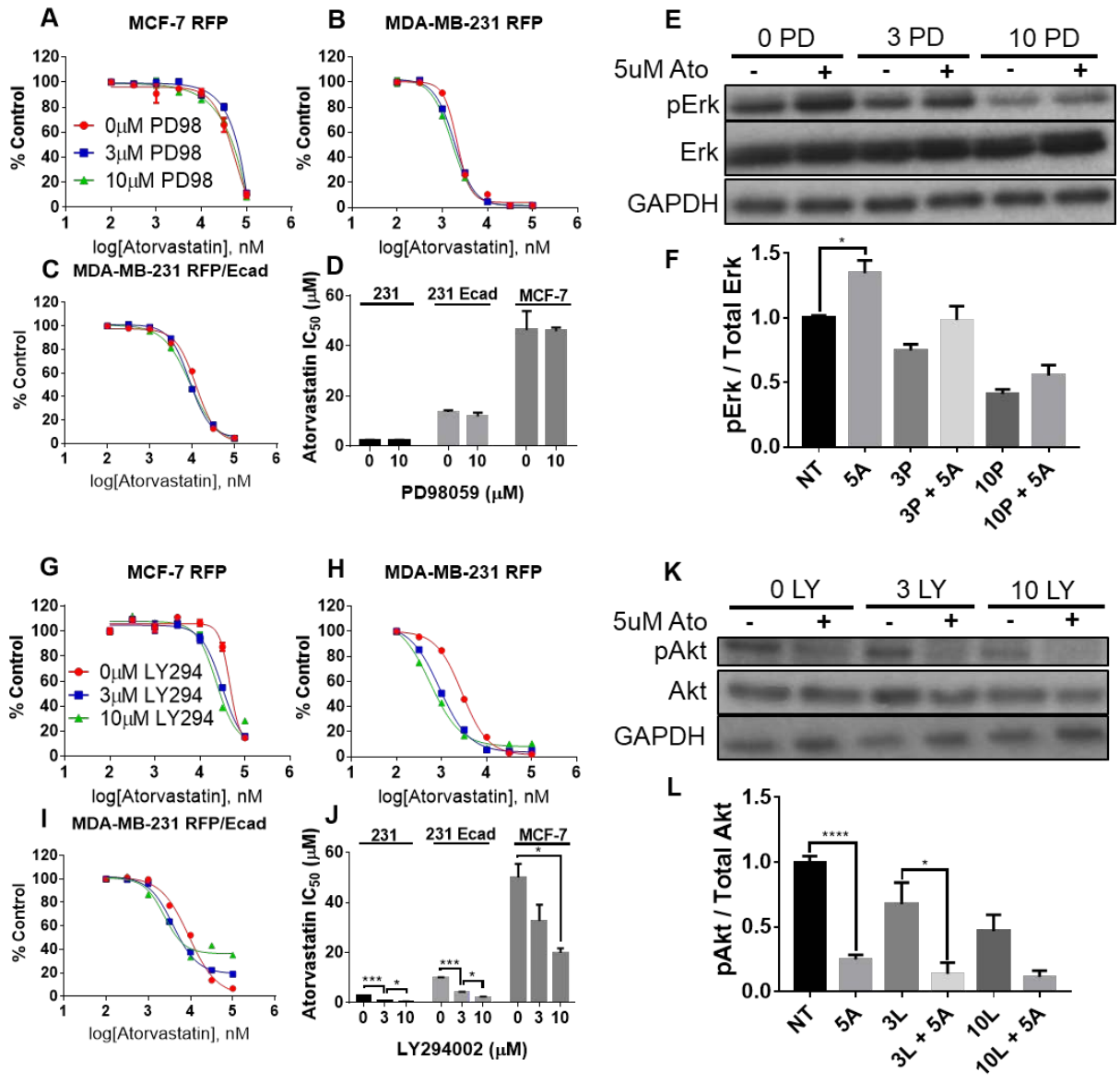


Figure 34. Inhibition of Akt but not Erk signaling is synergistic with atorvastatin

(A,G) MCF-7 RFP, (B,H) MDA-MB-231 RFP, and (C,I) MDA-MB-231 RFP/Ecad cells were cultured with atorvastatin and either (A-C) 0μM, 3μM, or 10μM PD98059 or (G-I) 0μM, 3μM, or 10μM LY294002 for 72 hours. (D,J) IC_{50} values for atorvastatin susceptibility were extrapolated from sigmoid curve fits of the dose response data. MDA-MB-231 RFP cells were treated with (E,F) 0μM, 3μM, or 10μM PD98059 or (K,L) 0μM, 3μM, or 10μM LY294002 with or without 5μM atorvastatin for 24 hours and (E,K) probed by western blot and (F,L) quantified by densitometry. * $P < 0.05$, *** $P < 0.001$, and **** $P < 0.0001$. All data are representative of at least three independent experiments.

6.4 SUPPLEMENTAL DATA

In order to test another lipophilic statin, we examined the ability of simvastatin to suppress growth of breast cancer cells. The lipophilicity and affinity of simvastatin and atorvastatin are comparable (Table 14, Table 15). We found that simvastatin demonstrated comparable efficacy to atorvastatin in MCF-7 RFP and MDA-MB-231 RFP cells (Figure 35A,B). Activation of simvastatin by conversion from its lactone form into its acid form did not alter its growth suppressive efficacy (Figure 35C,D), which suggests simvastatin lactone can be hydrolyzed in culture media. These data further emphasize the importance of lipophilicity in determining statin efficacy.

To confirm the growth suppressive effects of statins are due to its known mechanism of action (inhibition of HMGCR), we reduced HMGCR levels using siRNA transfection (Figure 36). We found that a knockdown of 50% at the protein level (Figure 36H,I) in MDA-MB-231 breast cancer cells was sufficient to suppress cell growth (Figure 36A-C) and also potentiated the efficacy of both atorvastatin and pravastatin by over an order of magnitude (Figure 36D,F,G). In contrast, sensitivity to doxorubicin, a chemotherapeutic used in the clinical management of breast cancer, was unchanged with HMGCR knockdown (Figure 36E,G).

The HMGCR siRNA data presented in Figure 36 are significant for three reasons. First, knockdown of HMGCR alone was sufficient to reduce cell growth by approximately 20%. Since the IC_{50} of atorvastatin in MDA-MB-231 RFP cells is approximately 2μM, the atorvastatin equivalent dose for a growth reduction of 20% would be approximately 800nM. Second, pravastatin, which previously demonstrated no efficacy up to concentrations of 100μM, was dramatically potentiated by knockdown of HMGCR (Figure 27, Figure 36). Finally, knockdown of HMGCR did not potentiate pravastatin by intrinsically reducing cell viability, as the potency of doxorubicin was unaffected by transfection.

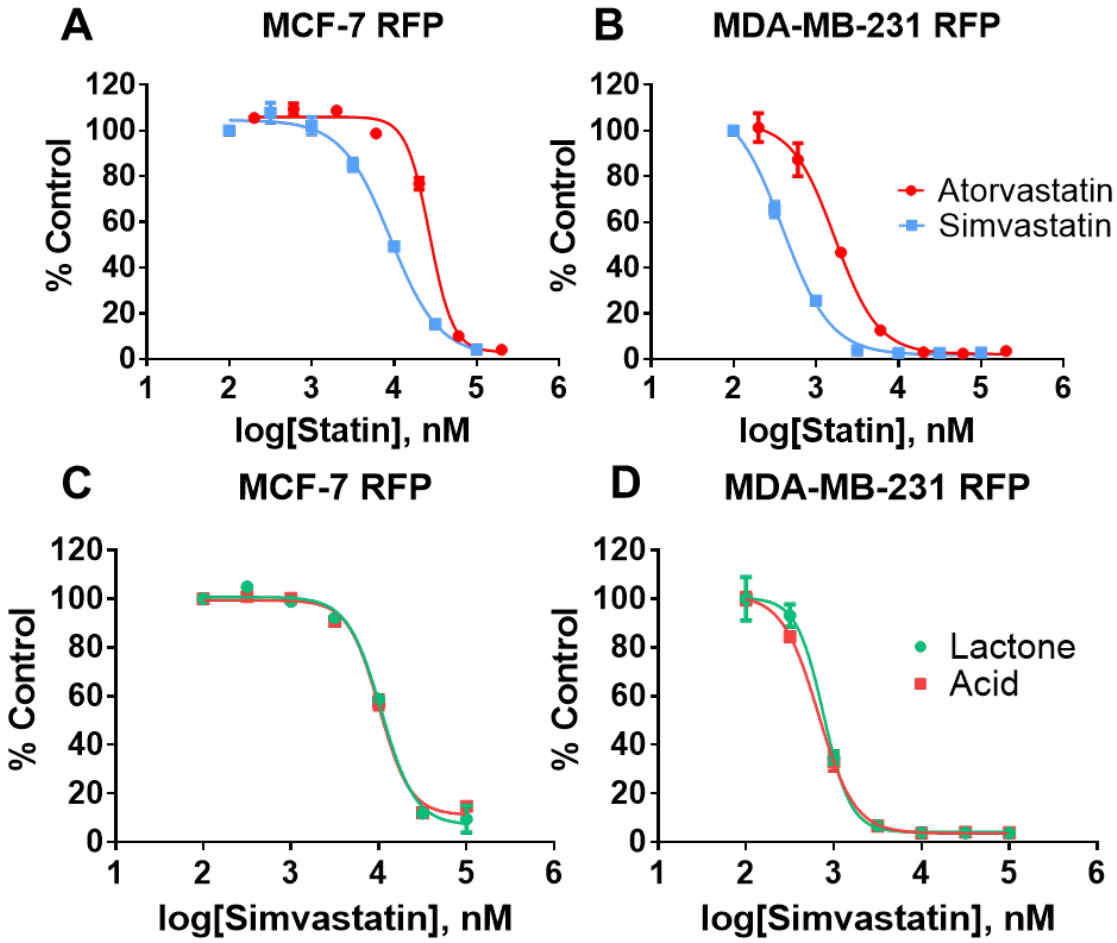
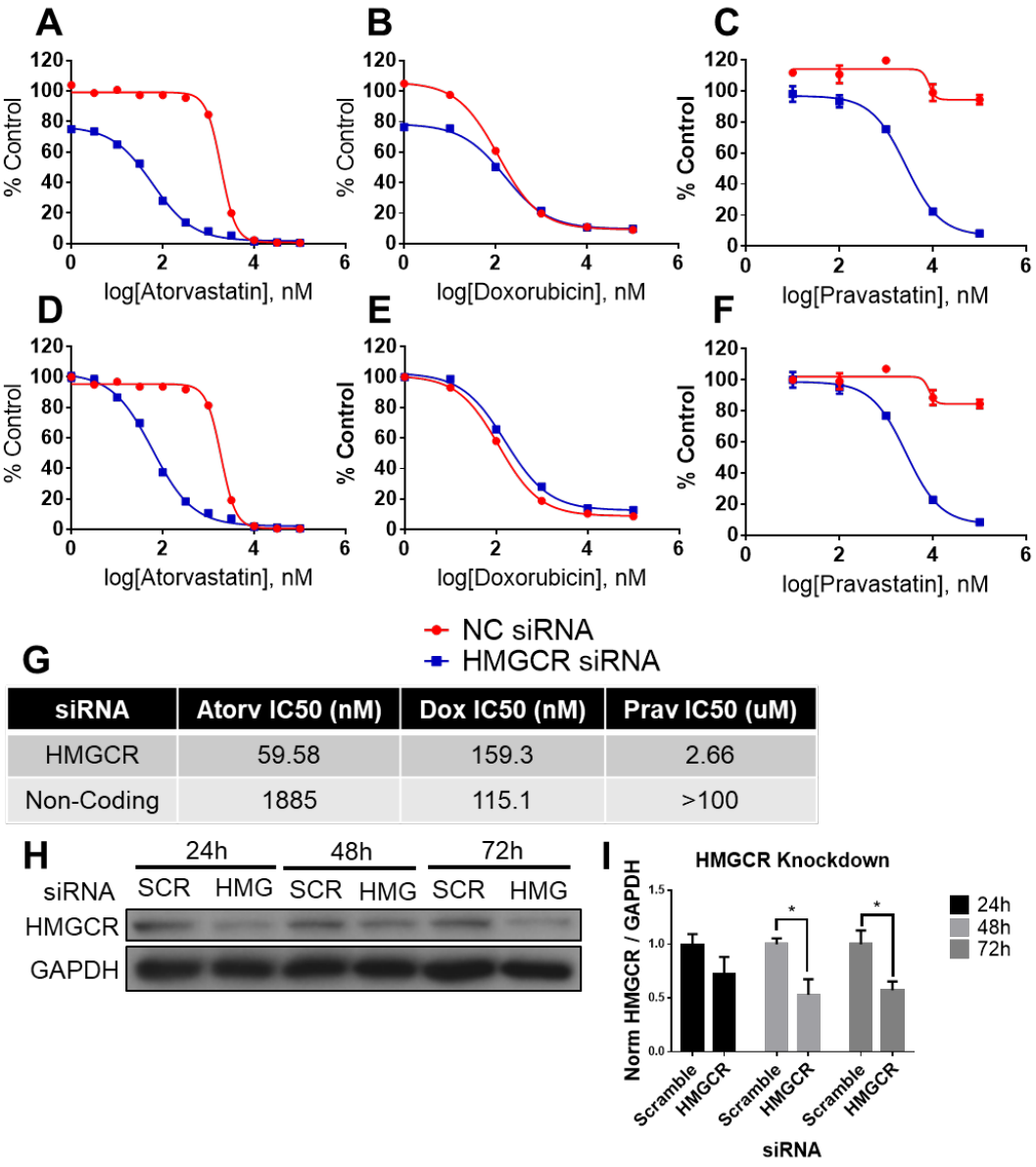


Figure 35. Simvastatin exhibits similar growth suppressive potency as atorvastatin

(A) MCF-7 RFP and (B) MDA-MB-231 RFP cells were cultured with atorvastatin or simvastatin for 72 hours, and cell number was determined by crystal violet staining. (C) MCF-7 RFP and (D) MDA-MB-231 RFP cells were cultured with simvastatin lactone (un-activated) or simvastatin acid (activated) for 72 hours, and cell number was determined by crystal violet staining. All data are representative of at least three independent experiments.



siRNA	Atorv IC50 (nM)	Dox IC50 (nM)	Prav IC50 (uM)
HMGCR	59.58	159.3	2.66
Non-Coding	1885	115.1	>100

Figure 36. HMGCR knockdown decreases cell growth and potentiates statin therapy

HMGCR was knocked down by siRNA treatment in MDA-MB-231 cells, and cells were subsequently treated with (A,D) atorvastatin, (B,E) doxorubicin, or (C,F) pravastatin for 72 hours. Data were normalized to the (A-C) non-coding RNA control and then further normalized to the (D-F) lowest dose of drug used. (G) IC50 values for atorvastatin (Atorv), doxorubicin (Dox), and pravastatin (Prav) were calculated based on sigmoid curve fits to the dose response data. (H) HMGCR immunoblotting 24, 48, and 72 hours after siRNA knockdown with (I) quantification by densitometry. * $P < 0.05$. All data are representative of at least three independent experiments.

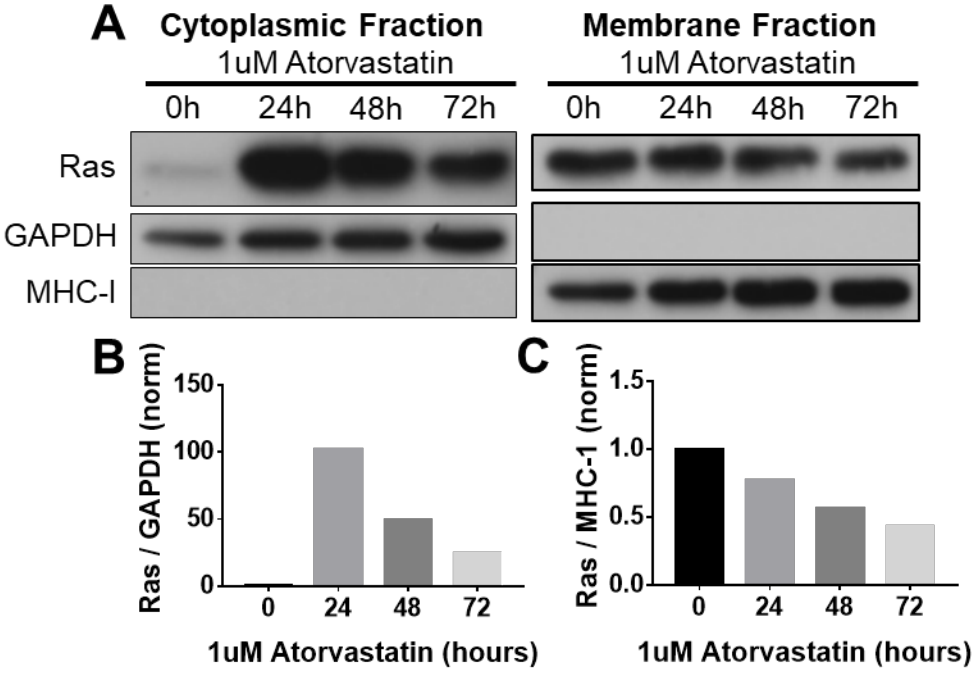


Figure 37. Ras localization in atorvastatin treated MDA-MB-231 RFP cells

MDA-MB-231 RFP cells were treated with 1μM atorvastatin for 0, 24, 48, or 72 hours, and protein was collected in cytoplasmic and membrane fractions and (A) probed by western blot. (B) Cytoplasmic Ras and (C) membrane Ras were quantified by densitometry. All data are representative of at least three independent experiments.

In order to determine the localization of Ras after atorvastatin treatment at a time point later than 48 hours, we carried out statin treatment to 72 hours. After prolonged statin treatment, we see degradation of cytoplasmic Ras (Figure 37A,B), as has been previously reported in the literature[932]. Moreover, we observe a continued reduction of membrane-bound Ras between the 48 and 72 hour time points (Figure 37A,C). These data suggest that prolonged treatment with atorvastatin results in the release of Ras from the membrane into the cytoplasm, where it is gradually degraded.

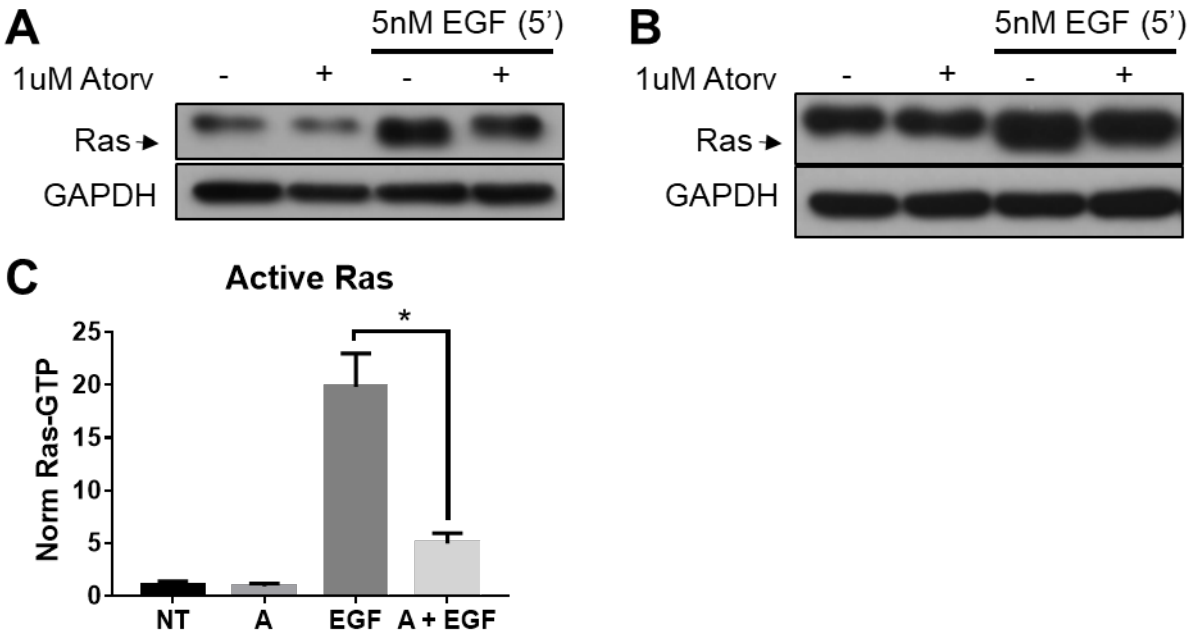


Figure 38. Atorvastatin pre-treatment reduces EGF-stimulated Ras activation

MDA-MB-231 RFP cells were treated with or without 1μM atorvastatin for 48 hours, and then cells were stimulated with 5nM EGF for 5 minutes. Activated Ras (Ras-GTP) was isolated from cell lysates, (A,B) probed by western blot, and (C) quantified by densitometry of the faster mobility fraction, indicated by the arrow. Atorv = Atorvastatin, NT = No treatment, A = 1uM Atorvastatin for 48 hours, EGF = 5nM EGF for 5 minutes. Error bars represent the SEM. * $P < 0.05$. All data are representative of at least three independent experiments.

While membrane localization of Ras has been previously shown to be correlated with activity[195], we assessed Ras activity after atorvastatin treatment directly by determining its binding affinity to its downstream partner Raf-1. After 48 hours of atorvastatin treatment, the ability of EGF to activate Ras was reduced by approximately 80% (Figure 38). In comparison, after 48 hours of atorvastatin treatment, the membrane fraction of Ras is reduced by approximately 50% (Figure 37). These data suggest that the membrane localization of Ras is directly linked to its activity. Moreover, atorvastatin can directly reduce EGF-mediated activation of Ras.

Finally, to further investigate crosstalk between the MAP Kinase and PI3K-Akt pathways, we determined the phosphorylation of Erk and Akt after treatment with the PI3K inhibitor LY294002 or the Mek1/2 inhibitor PD98059, respectively. While the PI3K inhibitor increased Erk phosphorylation in a dose-dependent manner, we saw no effect of the Mek1/2 inhibitor on Akt phosphorylation (Figure 39).

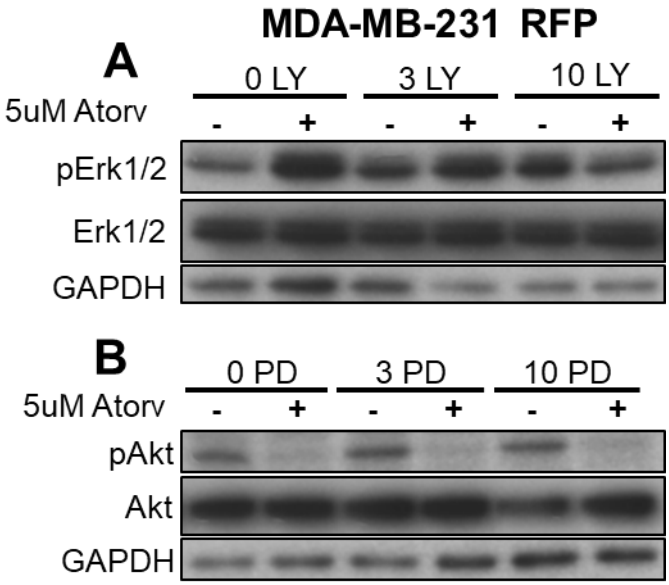


Figure 39. PI3K inhibition enhances Erk phosphorylation

MDA-MB-231 RFP cells were treated with or without 5μM atorvastatin supplemented with (A) 0μM, 3μM, or 10μM LY294002, an inhibitor of PI3 kinase or (B) 0μM, 3μM, or 10μM PD98059, an inhibitor of MEK for 24 hours. (A) pErk and total Erk or (B) pAkt and total Akt were probed by western blot. Importantly, the distinction being made is with increasing doses of either LY294002 or PD98059 (comparing lanes 1, 3, and 5). The effect of atorvastatin treatment (comparing lanes 1 & 2, 3 & 4, and 5 & 6) on Akt and Erk phosphorylation is the same as shown in Figure 6. All data are representative of at least three independent experiments.

6.5 CONCLUSIONS

This study adds to the previous study by examining the pharmacologic properties of statins that guide efficacy and the signaling pathways and cellular responses that result in the growth suppressive efficacy of statins. Importantly, not all statins are equally effective, with high affinity and lipophilic statins demonstrating the highest anti-tumor efficacy. Statins suppress cancer growth bi-directionally: by decreasing proliferation and by reducing cell survival. In part, statin efficacy is due to a reduction in prenylation and hence membrane localization of Ras, a critical node for signaling through downstream pathways to affect cell proliferation and survival. Specifically, statin treatment reduces signaling through the PI3K – Akt pathway, and further inhibition of this signaling pathway can potentiate statin efficacy. In contrast, we found that Erk activity was increased with atorvastatin treatment, suggesting crosstalk between these two signaling pathways.

The most relevant components of this study to the overarching hypothesis of this project are twofold. First, the differing potencies of statin drugs have important clinical implications. Our data suggest lipophilic statins may be more effective at suppressing micrometastatic outgrowth because they have increased uptake into cancer cells. For cancer patients already receiving statins for other conditions, such as hypercholesterolemia, changing to a more potent anti-cancer statin, such as atorvastatin, may provide a mortality benefit without adversely affecting their primary indication for statin therapy. Second, this study demonstrates that atorvastatin can reduce signaling through the Ras – PI3K – Akt signaling pathway. Previous studies in breast cancer have shown that Akt can regulate E-cadherin expression and induce EMT[228,933]. Since statins can reduce Akt activity, this finding suggests statins may reduce Akt-mediated EMT of breast cancer cells. Moreover, since E-cadherin directly provides survival signaling through the PI3K-Akt

pathway[914,934], statin therapy, while itself less effective in E-cadherin expressing cells[2], may partially reduce these survival signals and sensitize cells to other anti-tumor therapies.

The next section of this thesis will focus on attempts to identify alternative biomarkers that demarcate statin sensitive and resistant cells. The final experimental section of this thesis will test atorvastatin in sophisticated *ex vivo* and *in vivo* models of breast cancer metastasis to determine whether statins are indeed able to reduce the proliferation and outgrowth of these eventually mortal micrometastases.

7.0 BIOMARKER IDENTIFICATION FOR STATIN SENSITIVITY OF CANCER CELL LINES

7.1 PROLOGUE

This piece of work contributing to this thesis was published in Biomedical and Biophysical Research Communications at the start of 2018[3], which resulted from work I did with a graduate student in Dr. Benos's lab at the University of Pittsburgh. The majority of this chapter is previously published in that manuscript, and the corresponding reference can be found both at the beginning of this document and in the references section. Of note, the "Primary Data" section refers to a discussion of the data published in the body of the manuscript and the "Supplemental Data" refers to data published in the supplement of the manuscript.

In our previous work, we demonstrated that not all cancer cell lines are susceptible to statin-mediated growth suppression[2]. As such, a genetic signature of statin sensitivity would enable researchers and clinicians to focus on predicted sensitive and resistant cell lines, tissues, and patients for further mechanistic and clinical studies. Moreover, those predictions would identify candidate biomarkers and genes that play a role in tumor susceptibility or resistance to statins. Finally, this model may suggest a role for statins in anticancer therapy for patients with predicted statin-sensitive tumors.

In this work, we utilized transcriptome data from fourteen NCI-60 cancer cell lines and their sensitivity data to two statin drugs to produce a genetic signature identifying statin sensitive cells. We enriched these data with publicly available gene expression data from the National Cancer Institute (NCI-60) and the Cancer Cell Line Encyclopedia (CCLE), and with biomarker

discovery algorithms that can distinguish direct from indirect interactions in large datasets. We experimentally confirmed the validity of the identified biomarker signature in an independent set of cell lines, showed that a subset of TCGA tumors are predicted to display statin sensitivity, and demonstrated the biological viability of the predicted signature.

These data contribute significantly to this body of work for a couple reasons. First, this study demonstrates that statin susceptibility data can be converted into a biomarker signature which can successfully predict the sensitivity of novel cell lines. This is clinically relevant as biomarker signature data could be matched to that of the primary tumor to determine whether a patient may benefit from adjuvant statin therapy after primary resection. Finally, the combined bioinformatics-experimental approach described here can be used to generate biomarker sensitivity signatures for anticancer therapies and generate hypotheses of mechanism of action of drug sensitivity and resistance. In summary, this work takes the first steps towards identifying tumors that may benefit the most from statin therapy.

7.2 MATERIALS AND METHODS

Cell culture, statin treatment and cell proliferation assay. We selected seven pairs of cell lines from the NCI-60 cancer cell panel. These cell lines represent seven different major solid tumor types. For each site, we selected one cell line with low and one with high protein synthesis rate, as previously reported[2]. The selected cell lines - colon cancer (HCT-116, KM-12), ovarian cancer (IGROV1, OVCAR3), breast cancer (HS-578T, T47D), lung cancer (HOP-92, NCI-H322M), prostate cancer (PC-3, DU-145), melanoma (SK-MEL-5, MDA-MB-435), and brain cancer (SF-295, SF-539) - were cultured in RPMI 1640 medium (Life Technologies, Grand Island, NY),

supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS, Life Technologies) and 0.5% penicillin/streptomycin (Life Technologies) at 37°C with 5% CO2. Atorvastatin (Sigma-Aldrich, St. Louis, MO) and Rosuvastatin (Santa Cruz Biotechnology, Santa Cruz, CA) were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich; final concentration of 0.1% in RPMI 1640 medium) at a final concentration of 10 μM. The cells were seeded in 6-well plates at a density of 1×105 cells/ml and incubated overnight prior to treatment with 10 μM Atorvastatin, 10 μM Rosuvastatin, or 0.1% DMSO (DMSO served as solvent control). Three independent experiments were performed. Cell proliferation was quantified at 2, 4, and 6 days by direct cell counting with ScepterTM Handheld Automated Cell Counter (EMD Millipore, Billerica, MA) using ScepterTM Tips–60 μm sensor (EMD Millipore).

IC50 determination. Predicted statin-resistant (BT-474 and NCI-H2170) and sensitive (SK-MES-1 and SK-MEL-24) cell lines were cultured as above, and seeded in 24-well plates at a concentration of 0.5×105 cells/mL (500μL per well). The next day, cells were treated with 0.1 μM, 0.3 μM, 1 μM, 3 μM, 10 μM, 30 μM, and 100 μM atorvastatin (Sigma). Cells treated with 0.2% DMSO (the concentration of DMSO in the 100 μM atorvastatin treatment condition) served as the control. Three days after treatment, the cells were washed with PBS and fixed in 3.7% formaldehyde (F79-1, ThermoFisher Scientific) for 15 minutes. Next, the cells were stained with 0.5% w/v crystal violet (Sigma-Aldrich) for 10 minutes. Excess dye was washed extensively with tap water. The absorbed dye was released with 2% SDS. The supernatants were mixed thoroughly before transfer to a 96-well plate to be read at 560 nm using a Tecan SpectraFluor microplate reader (Tecan US, Durham, NC). IC50 values were determined by fitting a standard, four-parameter sigmoid curve to the data. All treatments were carried out in triplicate samples and data are representative of three independent experiments.

Immunofluorescence microscopy. Cultured BT-474, NCI-H2170, SK-MES-1 and SK-MEL-24 cells grown on coverslips in a 12-well plate were fixed with 3.7% formaldehyde (F79-1, ThermoFisher Scientific) for 15 min, and then permeabilized with 0.1% Triton-X-100 (Fisher Scientific, Pittsburgh, PA) made in PBS for 20 min. Next, non-specific proteins were blocked in 3% BSA for 45 min at RT. The cells were incubated with a monoclonal mouse antibody to human E-cadherin (1:500, 13-5700, Invitrogen) in a humidified container overnight at 4°C. After overnight incubation, coverslips were washed three times with 0.5% BSA for 5 minutes each and then were probed with an Alexa Fluor 488 goat anti-mouse IgG (1:200, Fisher Scientific) in the dark for 1 hour at RT. Following one 5-minute wash with 0.5% BSA, nuclei were stained with DAPI (1 μg/ml) in PBS for 15 min at RT, washed three times with PBS, and mounted in an aqueous-based mounting medium (gelvatol, recipe courtesy of U-Pitt Center for Biological Imaging). Images were captured with the 60X oil objective lens on an Olympus BX40 fluorescence microscope (Olympus Optical).

Computational Models. Mixed Graphical Models [1] (MGM) is a new method to learn the direct associations in a dataset con-sisting of both categorical and continuous data. MGM characterizes the joint distribution of the categorical and continuous variables under the assumptions of linear Gaussian continuous variables and multinomial categorical variables. To prevent overfitting a sparsity penalty, λ, is employed. In accordance with Sedgwick et al. [2], we utilize separate sparsity penalties for each type, and the MGM method is used without modification from this work. A common problem with using probabilistic graphical models for biomarker identification in highly structured data, like gene expression, is strong correlations between variables (genes). These correlations result in a lack of stability of the biomarkers as small changes in the data can result in changes amongst the selected biomarkers; and multiplicity of the predictive

signatures, since multiple signatures that each contains highly correlated features, can be equally predictive. To mitigate this problem, we developed the Tree-Guided Recursive Cluster Selection (T-ReCS) procedure [3], which performs feature selection at the cluster level, on clusters that are generated dynamically during the feature selection process. We refer the reader to [3] for full details of the T-ReCS algorithm.

7.3 PRIMARY DATA

7.3.1 Biomarker identification for statin sensitivity using statin growth inhibition data

Previous experiments have demonstrated that statins, including atorvastatin (Lipitor), inhibit the growth of a subset of NCI-60 cancer cell lines, and that a subset of statins show similar half-maximal inhibitory concentration (IC_{50}) values[908]. To test if the chemical properties of statins affect their inhibition of tumor cells, we cultured fourteen cancer cell lines from the NCI-60 collection, derived from seven organ types, in standard growth medium in the presence of a lipophilic (10 μM atorvastatin) or hydrophilic (10 μM rosuvastatin) statin. Importantly, in enzyme assays these statins have similar affinities for their enzyme target, HMG-CoA reductase (HMGCR), with IC50 values of 8.2nM and 5.4nM for atorvastatin and rosuvastatin, respectively[562]. We previously demonstrated that atorvastatin affected the growth of these cancer cell lines differentially; some cell lines displayed full or partial growth inhibition while others were completely insensitive to atorvastatin treatment[2]. In the current study, we have found that all the atorvastatin sensitive cell lines except HOP-92 are partially or fully resistant to rosuvastatin at the tested drug concentrations (Figure 40).

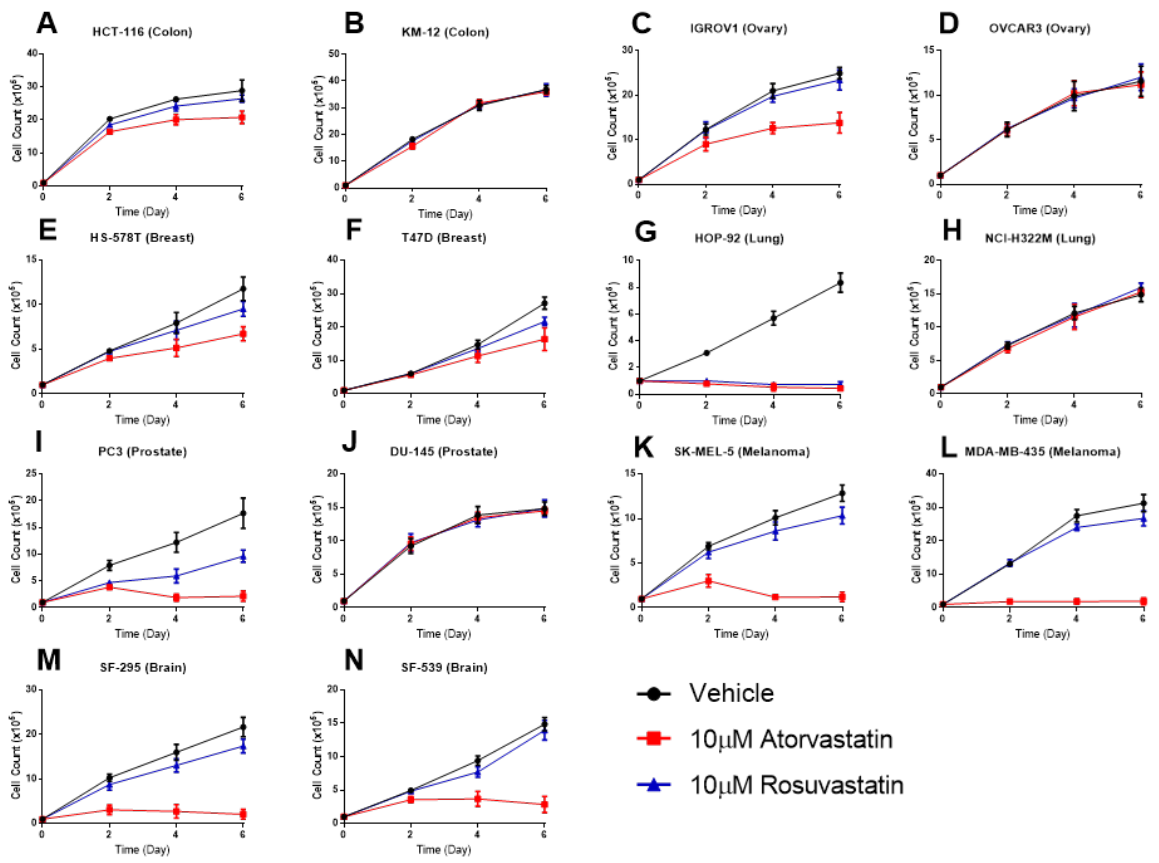


Figure 40. Growth rate of atorvastatin and rosuvastatin treated NCI-60 cancer cell lines

Colon cancer (A. HCT-116 and B. KM-12), ovarian cancer (C. IGROV1 and D. OVCAR3), breast cancer (E. HS578T and F. T47D), lung cancer (G. HOP-92 and H. NCI-H322M), prostate cancer (I. PC-3 and J. DU-145), melanoma (K. SK-MEL-5 and L. MDA-MB-435), and brain cancer (M. SF-295 and N. SF-539) cell lines from the NCI-60 cancer cell line collection were treated with 10 μM atorvastatin (pink line), 10 μM rosuvastatin (blue line), or DMSO vehicle control (black line) and cell growth was quantified at 2, 4, and 6 days through direct cell counting. The results are representative of three independent experiments. Error bars represent the standard deviation (n=3).

We utilized gene expression data from these fourteen NCI-60 cancer cell lines[908,935] and statin dose-response data (Figure 40) to produce a genetic signature distinguishing between atorvastatin and rosuvastatin sensitive and resistant cell lines. Specifically, our training set consisted of baseline gene expression data for the fourteen (non-treated) NCI-60 cell lines, and a measure of statin sensitivity as our response variable. We computed this response variable over a growth period of six days utilizing atorvastatin (ato), rosuvastatin (rosu), ato + rosu, or vehicle treatment on each cell line. From these data, dose-response curves were formed, depicting the number of (proliferating) cells at each time point. Using these curves, we found the ratio of the area under the dose response curve for the control and the area under the dose response curve for the treatment group, and used this number as a final response variable for each cell line.

To determine a genetic signature of statin sensitivity, the variables that were found to be associated to the sensitivity score measure from our graphical modeling methods were selected as important features. Specifically, we used three different methods for feature selection in this analysis. First, we used the "Atorva MGM" approach, which simply used the mixed graphical model (MGM) to produce an undirected graphical network from the NCI-60 data, and used all the genes adjacent to the statin sensitivity variable in the network as features. Nineteen genes were selected by this approach (Table 18). To determine the reliability of the selected genes, we employed a leave-one-out cross validation method using a support vector machine (SVM) regression model, and we obtained a cross validation error rate of 0.0082 for this set of features.

Table 18. Selected biomarkers for each identification method

Atorva-MGM	Combined-MGM	Atorva-TReCS
GABRA3	GABRA3	GABRA3
TM4SF18	TM4SF18	TM4SF18
ATP8B1		ATP8B1
CKAP4		CKAP4
IL13RA2	IL13RA2	
KRT7		KRT7
LAMA4		LAMA4
MACC1		MACC1
MISP		MISP
MMP14		MMP14
NFKBIZ		NFKBIZ
SCNN1A		SCNN1A
SLC16A4		SLC16A4
SLC27A2		SLC27A2
SLC6A8	SLC6A8	
SPINT2		SPINT2
TNFRSF10D	TNFRSF10D	
HIST1H1A	ANPEP	CD70
SRPX	GFPT2	CTSK
	PTGFRN	IFITM2
	RGS4	

Overlapping genes from all three methods are shown in red, and overlapping genes from two methods are shown in bold.

Our second approach was the "Combined" approach. Here, we used the MGM selected features from rosuvastatin on the NCI-60 dataset with the sensitivity scores from the rosuvastatin experiments. We combined them with the MGM selected features from the atorvastatin cell lines, and applied Tree-Guided Recursive Cluster Selection (T-ReCS) on the NCI-60 dataset to produce clusters of genes predictive of statin sensitivity. Following this step, we searched for those genes from the rosuvastatin MGM selected features that were in any cluster from the atorvastatin features selected. These were then utilized as the genetic signature. In total, nine genes were selected using this approach (Table 18), but the cross validation error rate was higher (0.1967).

Our final approach was the Atorva_TReCS approach. Here, we used the MGM features for the atorvastatin data as the original features for T-ReCS. Then, we ran our T-ReCS algorithm to produce clusters on these features. We then choose the final selected features for prediction, by simply taking a single gene from each cluster that had the highest correlation to the statin sensitivity variable. In total, seventeen genes were selected using this approach (Table 18) (as some genes from the original MGM selection appeared in the same cluster), with a cross validation error rate of 0.0285.

For each of these approaches, we used the corresponding gene expression signature to train a predictive linear regression model (a support vector machine (SVM) regression model trained via MATLAB's "fitrsvm" library) on the NCI-60 gene expression data. To validate the predictive power of this model, we obtained gene expression data from the Broad Institute's Cancer Cell Line Encyclopedia (CCLE)[936], and these data were used to predict an Atorvastatin sensitivity score for each of the cell lines in the CCLE database. We utilized the predictions from each of the three genetic signature selection approaches in the following manner. First, using the genes selected from each of the methods the top ten statin resistant cell lines were predicted using the model

trained on the NCI-60 database. Then, each of these cell line sets were combined and duplicates were removed resulting in 26 resistant cell lines. The same approach was employed on the set of 23 predicted statin sensitive cell lines. It is evident that the predictions by the three methods yielded a partial overlap for both the most statin sensitive and statin resistant cell lines. For this full data set with all of the scores for each of the three biomarker identification methods, the reader is directed to the supplemental material of the published manuscript[3].

7.3.2 Experimental Validation of Biomarker Predictions

Next, we assessed the fidelity of the predicted biomarkers by experimentally testing the statin sensitivity of yet untested cell lines. To reduce the number of cell lines to be experimentally tested, we clustered the predicted 23 statin sensitive and 26 statin resistant cell lines based on their whole transcriptome profiles, since cells displaying highly similar transcriptome profiles are likely to have similar biologic behavior. We performed principal component analysis on the full gene expression data for the predicted 26 statin resistant cell lines, and the cell lines were clustered according to their first two principal components using a k-means clustering with k = 10. The same approach was employed on the set of 23 predicted statin sensitive cell lines. These data are shown in the "supplemental information" section.

To test the atorvastatin sensitivity of representative cell lines from the predicted sensitive and resistant groups, we selected the NCI-H2170 (lung cancer-derived) and BT-474 (breast cancer-derived) predicted statin resistant; and the SK-MES-1 (lung cancer-derived) and SK-MEL-24 (melanoma-derived) predicted statin sensitive cell lines. To determine the IC50 of atorvastatin, we treated the cell lines with half log doses of atorvastatin, between 100nM and 100uM. As shown in Figure 25, cell lines predicted to be sensitive (SK-MES-1 and SK-MEL-24) were more sensitive

to atorvastatin than the cell lines predicted to be resistant (NCI-H2170 and BT-474) to it (Figure 41A, B), confirming the fidelity of the predicted statin-sensitivity gene expression signature.

Statin resistance correlates with cell membrane E-cadherin expression in cancer cell lines[2]. To determine the expression of E-cadherin in BT-474, NCI-H2170, SK-MES-1, and SK-MEL-24 cell lines we immunostained these cell lines for E-cadherin, as previously described[2]. We find that statin resistant cells with high mRNA for E-cad (BT-474 and NCI-H2170) have high membrane E-cadherin expression. In contrast, statin-sensitive cells with low mRNA for E-cad (SK-MES-1 and SK-MEL-24) have no detectable E-cadherin staining (Figure 41C-F). The uncovered E-cadherin expression pattern agrees with E-cadherin mRNA expression data deposited for these cell lines in Oncomine[937] and demonstrates that membrane E-cadherin is a marker of statin-resistant cells as we have found in previous studies[2]. Moreover these data further support the paradigm that tumor cells with an epithelial phenotype are more resistant to statin therapy, thus suggesting cells primed to undergo EMT and outgrow as mesenchymal masses would be selectively targeted by statins.

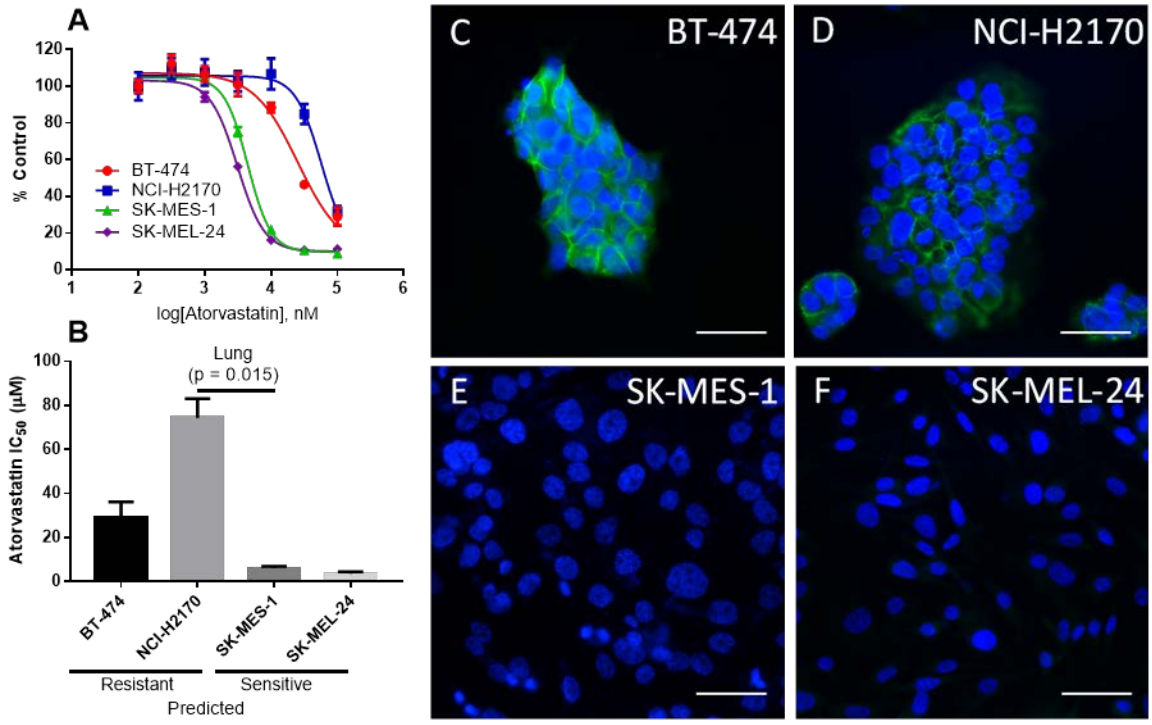


Figure 41. Statin sensitivity data correlates with predictions and membrane E-cadherin expression

(A) Dose response curves for Atorvastatin in predicted resistant (BT-474 and NCI-H2170) and predicted sensitive (SK-MES-1 and SK-MEL-24) cell lines. (B) Predicted resistant cell lines exhibited higher IC50 values to atorvastatin than predicted sensitive cell lines. Data are representative of three independent experiments. All experiments were carried out in triplicate (N=3). Error bars represent the standard error of the mean. Atorvastatin resistant cell lines (C) BT-474 and (D) NCI-H2170 display strong membrane E-cadherin (green) staining. In contrast, atorvastatin sensitive cell lines (E) SK-MES-1 and (F) SK-MEL-24 exhibited no E-cadherin staining. DAPI (blue) was used to counterstain the cell nuclei. Scale bar = 50μm.

7.3.3 Determination of Potential Combination Statin Therapies

To determine if there was potential for combination therapies with statins across cell lines, we analyzed publicly available pharmacological profiles from the Cancer Cell Line Encyclopedia and

the Sanger Center Genomics of Drug Sensitivity project. The CCLE data profiled 24 molecules across 504 cell lines[936], and the Sanger Center profiled 250 molecules across 549 cell lines[938]. Cell line sensitivity measurements for each molecule on each profiled cell line was computed using the IC50 value and the area under the dose response curve (AUC) for the CCLE data and the Sanger center data, respectively. These sensitivity measurements were compared to predict statin sensitivity on only those cell lines appearing in both the CCLE baseline gene expression dataset as well as the pharmacological profiling datasets, and correlation scores were produced (Table 19).

The main drugs with highest statistical significance are BRAF inhibitors (Dabrafenib, PLX-4720, SB590885), MEK inhibitors (Refametinib, Selumetinib), Bcl-2/Bcl-x/Mcl-1 inhibitors (TW37), NF-κB inhibitors (piperlongumine) and HSP-90 inhibitors (Elesclomol) (Table 19). In recent studies, simvastatin was shown to have additive or synergistic effects in combination therapy with BRAF-inhibitor, PLX-4720[939,940], which seem to depend on the mutational status of melanoma cells[940]. A MEK inhibitor was also shown to synergize with statin in a Drosophila model of lung cancer[941]. Thus, other inhibitors of the Ras-Raf-MEK-ERK pathway could also be tested for synergy with statins. Treatment with mitogen-activated protein kinase (MAPK) pathway targeted therapies (i.e., combined BRAF and MEK inhibitors) is the current standard of care for patients with BRAF-mutant advanced melanoma demonstrating the clinical relevance of MEK-inhibitors. However, response to these therapies in patients typically lasts less than a year, suggesting that further research is necessary for the better clinical management of these patients[942].

Table 19. Drugs predicted to synergize with statin therapy

Drug Name	Target	Correlation	P Value
Dabrafenib	BRAF	0.456976009	2.38E-18
PLX-4720	BRAF	0.376159659	4.97E-13
(5Z)-7-Oxozeaenol	TAK1	0.376014976	8.61E-13
SB590885	BRAF	0.340372121	4.32E-09
TW 37	BCL2, BCL-XL, MCL1	0.27305505	1.89E-06
Piperlongumine	NF-κB	0.226445462	0.000199099
Cisplatin	DNA crosslinker	0.233847531	0.000250235
Lestauritinib	FLT3, JAK2, NTRK1, NTRK2, NTRK3	0.218300548	0.000905096
Elesclomol	HSP90	0.206861021	0.002058482
Refametinib	MEK1, MEK2	0.183970201	0.004239963
Bleomycin	dsDNA break induction	0.183339449	0.004370418
Temsirolimus	MTOR	0.182232337	0.01021699
Nutlin-3a (-)	MDM2	0.176424024	0.010924768
Selumetinib	MEK1, MEK2	0.165658468	0.010924768
AZ628	BRAF	0.296949736	0.011550955
PD0325901	MEK1, MEK2	0.173101761	0.011550955
Serdemetan	MDM2	0.164726002	0.011550955
Trametinib	MEK1, MEK2	0.166312205	0.011550955
Docetaxel	Microtubule stabilizer	0.169092858	0.013420485
BX796	TBK1, PDK1 (PDPK1), IKK, AURKB, AURKC	0.167710541	0.013718152
Embelin	XIAP	0.157305764	0.016974867
FH535	PPARγ, PPARΔ	0.154672573	0.02034349
YK-4-279	RNA helicase A	0.158950401	0.026108609
Rucaparib	PARP1, PARP2	0.147699505	0.027477555
CI-1040	MEK1, MEK2	0.152967932	0.032836113
SN-38	TOP1	0.140036378	0.040080871
CHIR-99021	GSK3A, GSK3B	0.135343422	0.041404418

Pharmacological profiles from CCLE and Sanger Center drug sensitivity data were used to predict combination therapy with statins. Molecules with positive correlation and FDR Q-Value < .05 were included. For CCLE profiles, IC50 was used as a proxy for sensitivity, and for Sanger center profiles, the area under the dose-response curve (AUC) was used.

HSP-90 family members can both suppress or contribute to tumor invasiveness. Also, cell surface HSP-90 contributes to metastasis formation[943], while the mitochondrial HSP-90, TRAP-1 suppresses tumor cell invasiveness and plays a role mediating the switch from OxPhos to aerobic glycolysis[944]. Indeed, recent studies have also demonstrated that simvastatin synergizes with the HSP-90 inhibitors, 17-DMAG[940] or 17-AAG[939] in inhibiting melanoma cell proliferation.

Although they have not yet been tested in combination with statins the other main identified mechanisms are also of potential interest for statin co-therapy. Bcl-2 and Bcl-xL can promote cell migration, invasion and metastasis perhaps by their promotion of mitochondrial ROS formation[945]; In contrast, pro-apoptotic Bcl-2 family members, Bax and Bak attenuate cell migration and metastasis formation[945]. The selective Bcl-2 inhibitor ABT199/ Venetoclax is FDA approved and used for treating relapsed/ refractory CLL with 17p deletion. Similarly, the essential role of NF-kB in epithelial-mesenchymal transition (EMT), the main initial event in metastasis development, is well recognized[946,947], and its inhibitors can delay breast cancer invasiveness[948]. These data argue that if they are expressed to a sufficient degree in a given cell line, the inhibition of both Bcl-2 and/ or NF-kB may synergize with the tumor growth-inhibitory function of statins.

7.3.4 Limitations of this Computational Approach

While this study generated a computational framework for approaching a genetics-based approach for predicting statin sensitivity, our current approach is limited by the data upon which it was built. First, the vast majority of available large datasets (e.g., TCGA) are derived from primary tumors; this skews the analysis towards relatively well differentiated (epithelial, E-cadherin-positive) cells that are not epidemiologically linked to reduction in mortality from cancer which is more related to disseminated/metastatic disease (usually mesenchymal, E-cadherin low/absent). Thus, the

tumor selection is not enriched in the tumor cells that are postulated as the direct targets for statins. Second, since the gene expression data are derived from whole tumors, tumor heterogeneity is expected to further obscure the statin sensitivity/resistance signal. Third, gene expression data is inherently limited by high dimensionality (i.e., few cell lines per tumor type compared to the large number of genes/variables), which reduces statistical power. Moreover, predicted sensitivity genes were not examined at the genetic level to determine whether altered expression influences statin efficacy. Finally, predicted synergistic drug combinations with statins were not tested experimentally in these cell lines, although some have been already confirmed in the literature. These limitations are beyond the scope of this work and will be addressed in subsequent studies.

7.4 SUPPLEMENTAL DATA

In order to determine genes that would denote sensitivity to atorvastatin, rosuvastatin, or both, we employed the Atorva-MGM (Figure 42), Combined-MGM (Figure 43), and Atorva-TReCS computational algorithms (Figure 44). As stated in the main text, nineteen, nine, and seventeen genes were identified by each of these three approaches, respectively. There were two overlapping genes found using all three approaches (Table 18) which are GABRA3 and TM4SF18. GABRA3 is $GABA_A$ receptor alpha 3, which is typically only found in adult brain but has also been found in cancers of lung and breast[949,950]. Interestingly, GABRA3 expression in lung cancer was found to be expressed more highly in low grade tumors[950] whereas in breast cancer high expression correlated with worse survival[949]. TM4SF18 is a Transmembrane-4-L-six-family protein that is found mostly in adult kidney, but its paralog TM4SF1 and other family members have been shown

to be upregulated in many cancers[951]. These data suggest that the genes extracted from all three of these computational methods have been previously shown to be relevant in cancer aggressiveness.

7.4.1 Selection of statin sensitive tumors from the TCGA breast cancer samples

The experimentally confirmed gene expression signatures allow the selection of those subsets of primary tumors or metastases that may be sensitive to statin treatment. To this end, we have applied the biomarker search for transcriptome profiles obtained from breast cancer samples that are deposited in the Cancer Genome Atlas (TCGA)[952]. We find that a small subset of primary breast tumors is predicted to be sensitive to statins while many of them are not (Figure 45). We find no significant correlation with tumor stage, age, race, presence or absence of metastasis (p=0.590, p=.101, p=.290, p=.275, respectively) (Table 20).

Subsequently, we have repeated the procedure for all tumor types in TCGA, finding overall similar trends. For the full complement of tumor type analyses, the reader is referred to the published manuscript's supplemental information document[3]. These data suggest that statin sensitivity is multifactorial and thus sophisticated biomarker signatures are needed in order to accurately predict the statin sensitivity of a particular tumor. Furthermore, while no correlation between statin sensitivity and metastasis was uncovered, the transcriptome profiles in the TCGA are derived from the primary tumor and thus may not be reflective of the rare cells that metastasized in those patients[952].

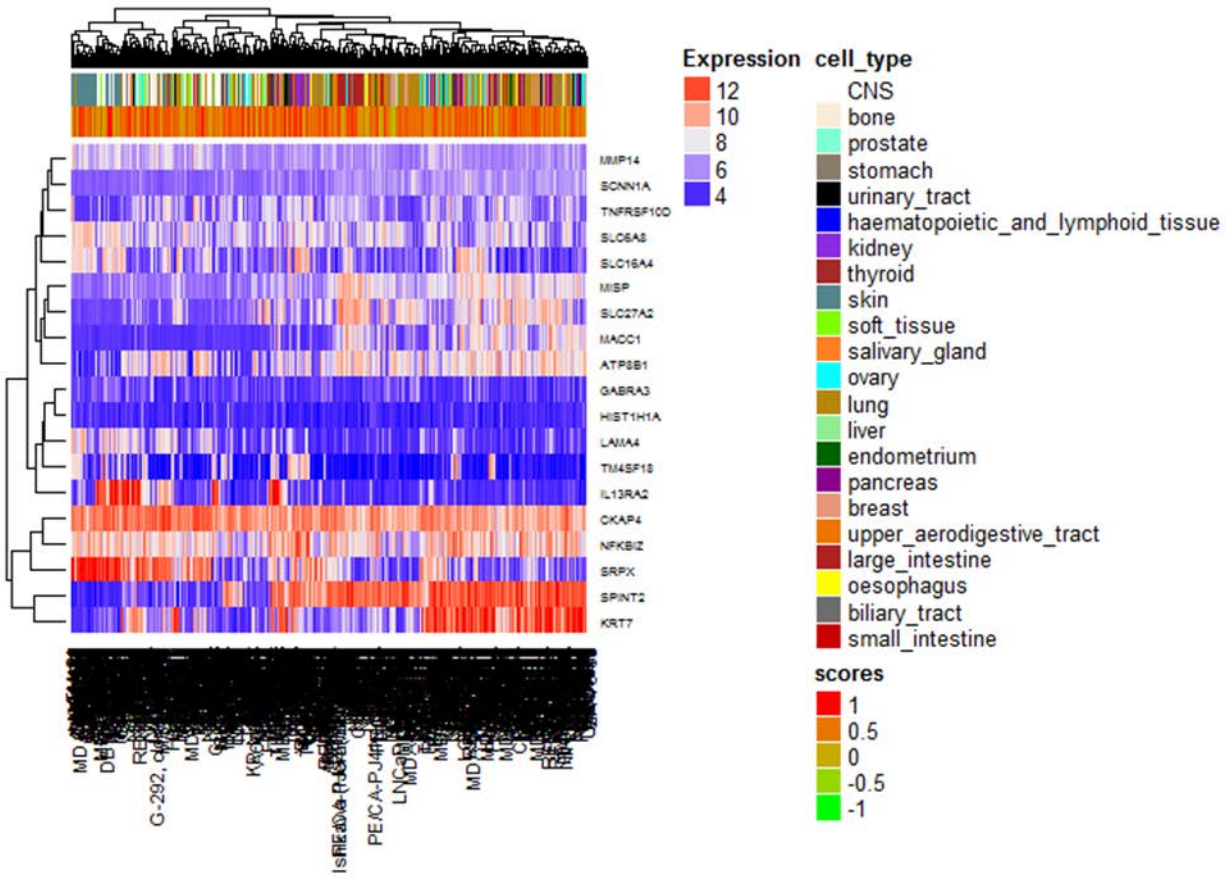


Figure 42. Potential biomarkers of atorvastatin sensitivity using Atorva-MGM

The CCLE cell lines' tissue of origin, mRNA expression levels (4-12) of potential biomarker genes and scores for atorvastatin sensitivity (1 to -1) are shown using the Atorva-MGM method.

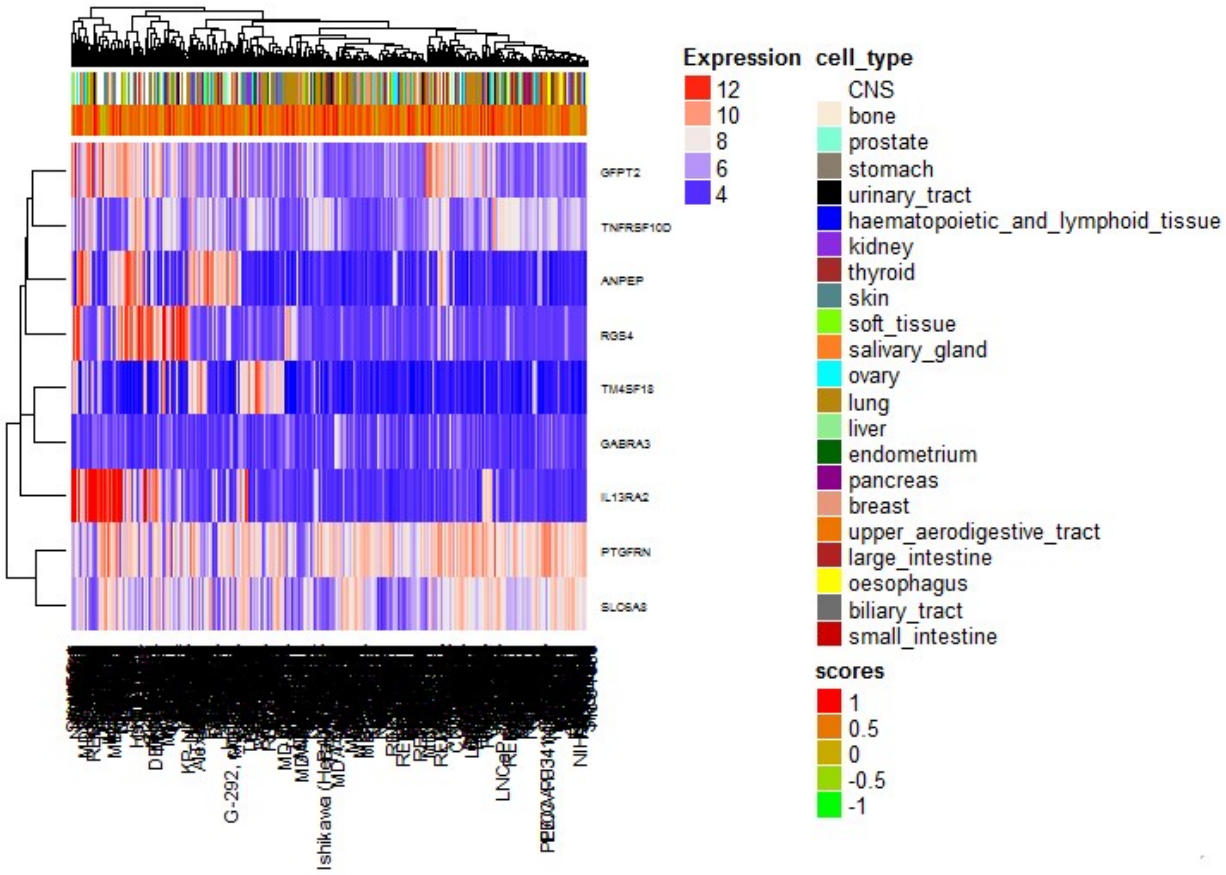


Figure 43. Potential biomarkers of atorva- and rosuva-statin sensitivity using combined-MGM

The CCLE cell lines' tissue of origin, mRNA expression levels (4-12) of potential biomarker genes and scores for atorvastatin sensitivity (1 to -1) are shown using the combined-MGM method.

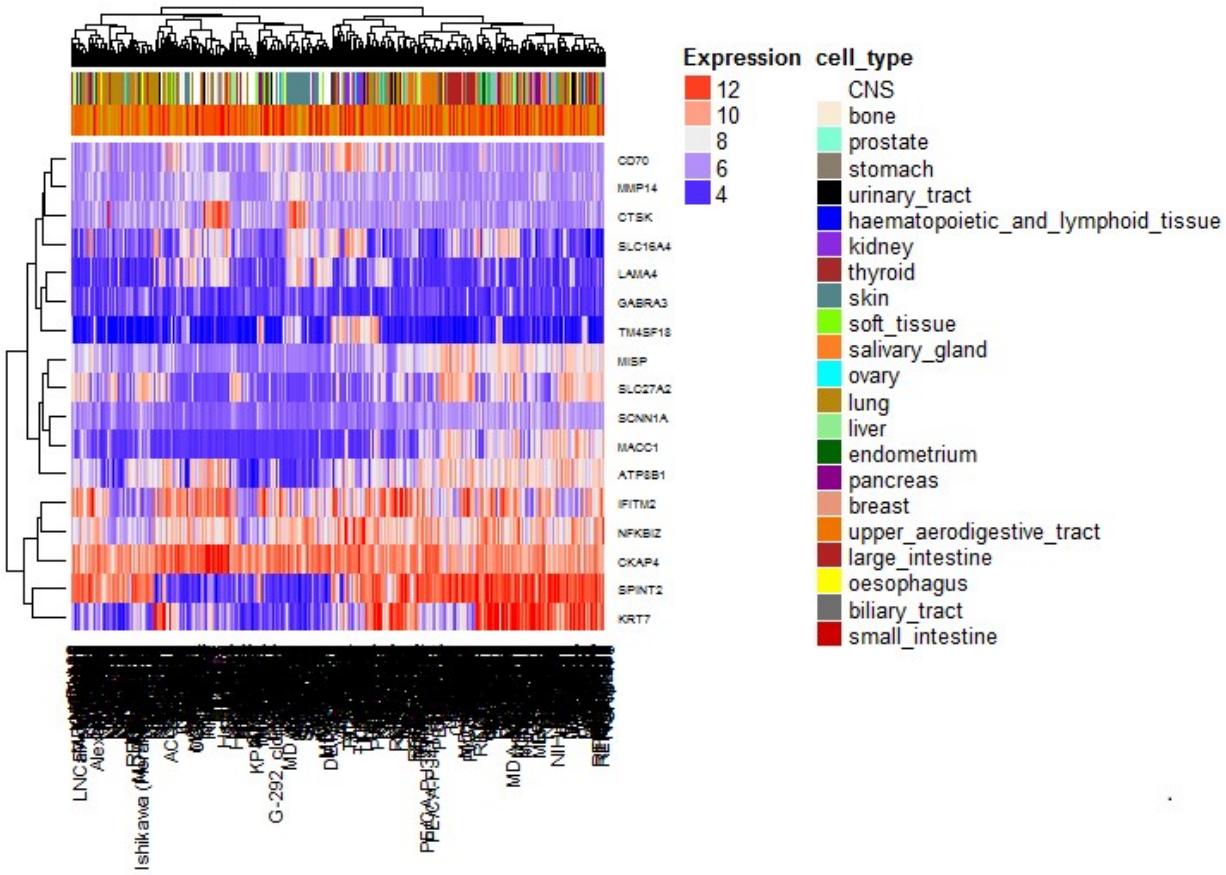


Figure 44. Potential biomarkers of atorvastatin sensitivity using Atorva-TReCS

The CCLE cell lines' tissue of origin, mRNA expression levels (4-12) of potential biomarker genes and scores for atorvastatin sensitivity (1 to -1) are shown using the Atorva-TReCS method.

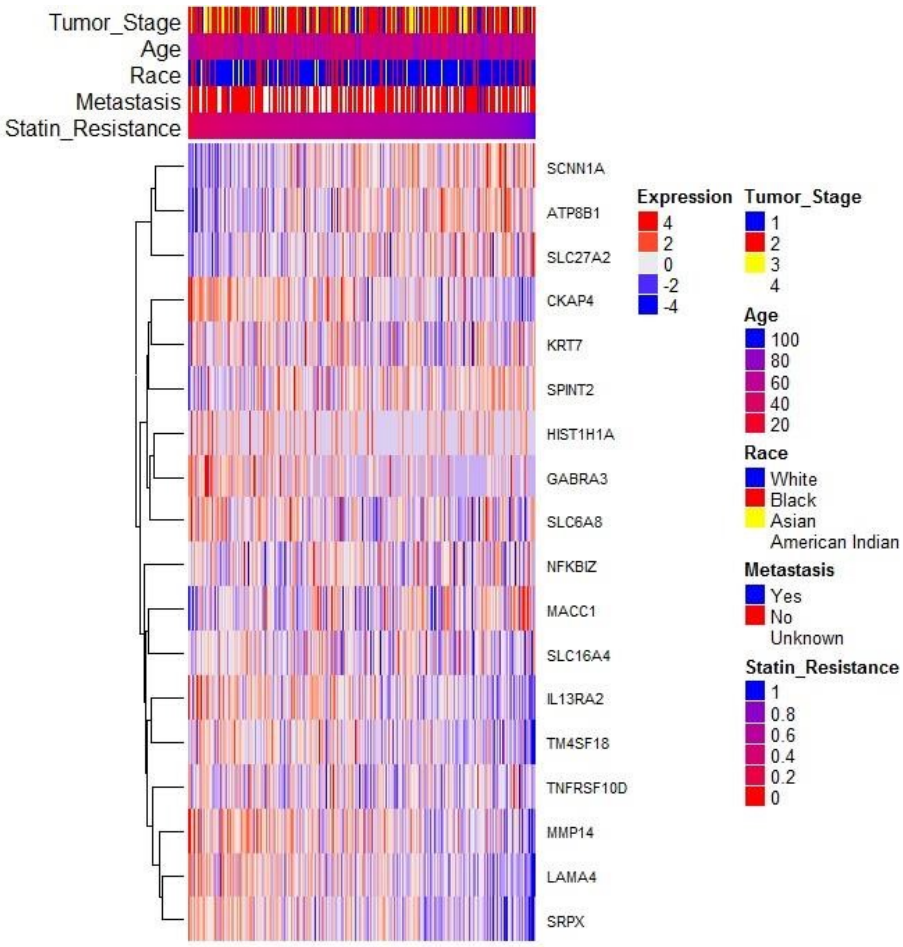


Figure 45. Predicted statin sensitivity of breast cancers deposited in TCGA

Transcriptome profiles of breast cancer samples deposited in TCGA were used as input into our statin sensitivity predictive model. Clinical covariates showed no significant relationship with statin sensitivity.

Table 20. Correlations of clinical covariates with predicted statin sensitivity

Tumor Type	COAD	LUAD	OV	PRAD
Metastasis	**1.59E-02**	NA	NA	NA
Gender	7.13E-01	4.60E-01	NA	NA
Age	9.51E-01	5.55E-02	**6.62E-03**	3.14E-01
Tumor Stage	3.53E-01	6.61E-01	2.18E-01	NA
Weight	7.08E-01	NA	NA	NA
Race	6.51E-02	3.95E-01	5.15E-01	3.73E-01
Tumor Type	**SKCM**	**GBM**	**KIPAN**	**LAML**
Metastasis	2.55E-01	NA	NA	NA
Gender	2.39E-01	1.33E-01	**5.23E-04**	8.47E-01
Age	2.87E-01	6.41E-01	9.93E-01	**1.25E-02**
Tumor Stage	6.79E-01	NA	**1.30E-11**	NA
Weight	9.54E-01	NA	**1.40E-02**	NA
Race	5.44E-01	5.19E-01	2.36E-01	2.14E-01

Correlations are shown as p-values with the null hypothesis of zero correlation. Statistically significant non-zero correlations with $p < .05$ are bolded. Columns refer to different tumor types from the TCGA, and rows refer to the clinical covariate. COAD = Colon Adenocarcinoma; LUAD = Lung Adenocarcinoma; OV = Ovarian Carcinoma; PRAD = Prostate Adenocarcinoma; SKCM = Cutaneous Melanoma; GBM = Glioblastoma Multiforme; KIPAN = Pan-Kidney Carcinoma; LAML: Acute Myeloid Leukemia.

7.4.2 Gene Ontology Enrichment Analysis

To validate the biological significance of the predictive biomarkers we performed gene ontology enrichment analyses using the Database for Annotation, Visualization and Integrated Discovery (DAVID)[527]. Three gene lists were submitted to the resource, representing the three major gene clusters (consisting of more than two genes) that we identified by the Atorva_T-ReCS approach (Tables 21-23). We only used the Atorva_T-ReCS approach for this analysis because unclustered predictive biomarkers tend to have a single representative from each pathway (since expression of genes in the same pathway tend to be correlated or anti-correlated), and thus ontological analysis would be unable to recognize important terms or important pathways. Through T-ReCS clustering, we mitigate this problem by finding multiple co-expressed genes in a single signature (see Materials and Methods).

Table 21. Ontology of cluster one from identified biological signature

GO Term	Relevant Genes	Corrected P-Value
Axonal transport of mitochondrion	UCHL1, NEFL	0.13939585
Axon cytoplasm	UCHL1, NEFL	0.250536938
Extracellular space	CXCL5, CD70, IL13RA2	0.439228308
Extracellular region	CXCL5, MMP1, IL13RA2	0.45323271
Myelin sheath	UCHL1, NEFL	0.466479946
Rheumatoid arthritis	CXCL5, MMP1	0.473252751
Cell-cell signaling	CXCL5, CD70	0.959289219

Gene ontology terms from cluster one of genes from the Atorva-T-ReCS identified biological signature. P-Value correction for multiple testing was applied using the Bonferroni correction method.

Table 22. Ontology of cluster two from identified biological signature

GO Term	Relevant Genes	Corrected P-Value
Extracellular space	CTSK, EREG, IL18, GAL, TNFAIP2	0.260167934
Angiogenesis	EREG, IL18, TNFAIP2	0.647369645
SNARE binding	VAMP8, TNFAIP2	0.66437542
Extracellular region	CTSK, EREG, IL18, GAL	0.759814148
Inflammatory response	TNFRSF10D, IL18, GAL	0.764152784
Rheumatoid arthritis	CTSK, IL18	0.801237029
Positive regulation of smooth muscle cell proliferation	EREG, IL18	0.814152797
Legionellosis	IL18, EEF1A2	0.861642906

Gene ontology terms from cluster two of genes from the Atorva-T-ReCS identified biological signature. P-Value correction for multiple testing was applied using the Bonferroni correction method.

Several ontological terms related to the submitted gene lists have evidence of association to statins in the literature. The two major ontological terms from cluster one (Table 21) refer to axonal transport of mitochondria and axon cytoplasm. Though there is no evidence for statins directly acting upon axons, their inhibition of the cholesterol synthesis pathway has been shown to delay axonal growth[953]. From cluster two (Table 22), statins have been shown to have biphasic effects on angiogenesis[713], and they have anti-inflammatory properties through their reduction in quantity of circulating C-reactive protein (CRP)[954]. Also, though it is not yet clear if statins have a direct effect on SNARE protein binding, we note that statins may affect the release of insulin, in which SNARE proteins also have a role[955]. Cluster three (Table 23) depicts two main terms: cell adhesion/migration and extracellular matrix organization. It has been shown that statins affect the adhesion related expression of selectin and VCAM-1[956], and they further inhibit migration of cell lines, and in fact this may relate to their anti-inflammatory properties[957,958]. In addition, statins

have been shown to modulate the metabolism of the extracellular matrix to prevent fibrosis by inhibiting connective tissue growth factor (CTGF) production[959].

Table 23. Ontology of cluster three from identified biological signature

GO Term	Relevant Genes	Corrected P-Value
Cell adhesion	LAMA4, LPXN, SRPX, TNC, POSTN, CDH2, ITGB3, THBS1, ENG, FN1, PCDH18, THY1	9.06E-08
Focal adhesion	LPXN, TNC, HSPA1A, CDH2, ITGB3, ENG, PLAU, THY1	1.81E-04
Extracellular matrix	LAMA4, SERPINE2, TNC, SERPINE1, POSTN, THBS1, FN1	2.46E-04
Extracellular matrix organization	LAMA4, TNC, SERPINE1, POSTN, ITGB3, THBS1, FN1	2.58E-04
Extracellular space	SERPINE2, TNC, SERPINE1, CD109, AXL, POSTN, DPYSL3, THBS1, ENG, PLTP, PLAU, FN1	2.64E-04
Cell surface	SRPX, CD109, AXL, VAMP5, ITGB3, THBS1, ENG, PLAU, HLA-DRA	2.71E-04
Extracellular region	INHBA, LAMA4, CHRDL1, SERPINE2, GLIPR1, TNC, SERPINE1, HIST1H3C, THBS1, PLTP, PLAU, FN1	8.55E-04
Regulation of cell migration	LAMA4, SERPINE2, DPYSL3, ITGB3, THY1	0.00136393
ECM-receptor interaction	LAMA4, TNC, ITGB3, THBS1, FN1	0.002847294
Negative regulation of plasminogen activation	SERPINE2, SERPINE1, THBS1	0.003599381

Gene ontology terms from cluster three of genes from the Atorva-T-ReCS identified biological signature. P-Value correction for multiple testing was applied using the Bonferroni correction method.

Overall, this demonstrates the relevance of the genes in our predicted signature to the mechanisms underlying the physiological effect of statin.

7.5 CONCLUSIONS

In this work, we have utilized several feature selection approaches to identify a predictive genetic signature of statin sensitivity from RNA-Seq data from TCGA. We applied this signature to several cancer cell lines from the NCI-60 database and externally validated the sensitivity predictions, confirming all four of our experimental predictions. We then applied our predictive signature to the TCGA-Breast Invasive Carcinoma project and found a subset of tumor samples predicted to be sensitive to statins, and we further validated our predictive signature through an ontological analysis for relevance of the selected genes.

These results contribute to this overall body of work by suggesting that genetic signatures can be used to determine tumors that may be responsive to statin therapy. Importantly, many of these predictions are made using specimens derived from primary tumors, and as such may not be reflective of the genetics of the metastatic cells. However, this study lays a framework for developing more sophisticated genetic signatures of metastatic cancer susceptibility to statin therapy. This will be critical for patient stratification, namely selecting those patients whom will benefit from adjuvant statin therapy after primary tumor resection.

In summary, we have developed a combined algorithm-experiment blueprint that is able to generate biomarker sensitivity signatures for anticancer therapies, as exemplified here by identifying an initial biomarker signature for statin sensitivity in human cancer cell lines and tumors. This signature was validated both in terms of biological sensibility and by experimental validation, and it confirms many recent physiological effects of statins as shown through our ontological analyses. Future studies will be aimed at validating these results in sophisticated *ex vivo* and *in vivo* models of cancer metastasis and testing predicted synergistic drug combinations.

8.0 STATINS INHIBIT OUTGROWTH OF BREAST CANCER METASTASES

8.1 PROLOGUE

The fourth piece of work contributing to this thesis is currently in review at British Journal of Cancer[960]. It is the second of the two submitted first author publications that have resulted from my thesis work. The majority of this chapter is taken directly from that manuscript, and the corresponding reference can be found both at the beginning of this document and in the references section. Of note, the "Primary Data" section refers to a discussion of the data published in the body of the manuscript and the "Supplemental Data" section refers to data published in the supplement of the manuscript.

In this work, we investigated the growth suppressive effects statins in 2D *in vitro*, 3D *ex vivo*, and *in vivo* models of breast cancer metastasis. We found that statins exhibit similar anti-tumor properties in breast cancer 2D co-culture with primary human hepatocytes. Moreover, atorvastatin is able to block LPS/EGF stimulated outgrowth of dormant tumor cells in a 3D *ex vivo* microphysiological system (MPS) model of breast cancer metastases in the liver. Finally, two independent mouse models of spontaneous breast cancer metastasis to the liver and lung, respectively, demonstrate that atorvastatin can suppress the growth of metastatic but not primary tumor cells.

These data are a significant foundation to this body of work for two main reasons. First, this section demonstrates the ability of atorvastatin to block stimulated outgrowth of dormant tumor cells. This is important in the context of reducing breast cancer recurrence, as many clinically detectable macrometastases often begin as dormant micrometastases[961]. Second, and

most importantly, we demonstrate that atorvastatin exhibits divergent effects on primary and metastatic tumor cells. While atorvastatin does not affect proliferation of primary tumor cells, it decreases proliferation of metastatic tumor cells. The central hypothesis of this thesis proposes that statin suppression proliferation and emergence of dormant micrometastases is responsible for the clinically observable reduction in breast cancer recurrence and mortality in women with breast cancer. The data discussed below greatly support this hypothesis because they demonstrate that atorvastatin can both suppress dormant tumor cell emergence and reduce proliferation at the metastatic site.

8.2 MATERIALS AND METHODS

Human Cells: Use of human cells were adjudicated as exempted by the University of Pittsburgh IRB, and all animal investigations were approved by the Pittsburgh VA Health System IACUC.

Reagents: Atorvastatin (PHR-1422) and rosuvastatin (SML-1264) were obtained from Sigma Aldrich, USA. Atorvastatin and rosuvastatin were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 50mM. PD98059 (S1177, SelleckChem) was reconstituted in DMSO at a concentration of 20mM. LY294002 (S1105, SelleckChem) was reconstituted in DMSO at a concentration of 10mM. Lipopolysaccharide (LPS, Sigma) and mouse epidermal growth factor (EGF, Corning) were reconstituted in sterile deionized water at concentrations of 1mg/ml and 50μg/mL respectively.

Cell Sourcing and Cell Culture: MCF-7 red fluorescent protein (RFP), MDA-MB-231 RFP, and MDA-MB-231 RFP/Ecad were previously transfected and stably selected[45]. These cell lines will be referred to in the results and discussion without inclusion of the RFP suffix. All cells

were maintained in RPMI 1640, GlutaMax Supplement (Gibco, ThermoFisher) supplemented with 10% heat inactivated fetal bovine serum (HI-FBS, Gemini Bioproducts) and 0.5% penicillin-streptomycin (Gibco, ThermoFisher), henceforth referred to as RPMI. Puromycin (Gibco, ThermoFisher) and G418 (Teknova) were used to maintain expression cassettes, at the following concentrations: 1μg/mL puromycin (MCF-7 RFP), 5μg/mL puromycin (MDA-MB-231 RFP), 900μg/mL G418 (MDA-MB-231 RFP/Ecad). Antibiotic selection media was removed at least 24 hours prior to beginning experiments. Primary human hepatocytes and non-parenchymal cells were obtained as isolates from excess pathology specimens at the University of Pittsburgh Medical Center (UPMC) as part of a National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)-funded Liver Tissue and Cell Distribution System run by Dr. David Gellar and funded by NIH contract #HHSN275201700005C. Hepatocytes were isolated by collagenase perfusion for subsequent distribution to investigators. Non-parenchymal cells were further isolated by density centrifugation using a percoll gradient, as previously described[844].

Statin IC_{50} Curve Determination: Cancer cells were seeded in 24-well plates at a concentration of $5x10^4$ cells/mL in a volume of 500μL. The next morning, cells were treated with atorvastatin in half log doses between 100nM and 100μM. The vehicle control treatment was 0.2% DMSO. After 72 hours of treatment, treatment solutions were aspirated and cells were fixed with 3.7% formaldehyde (F79-1, ThermoFisher) for 15 minutes. After fixation, cells were incubated with 0.5% w/v crystal violet for 10 minutes and excess dye was removed by copious washing with tap water. The absorbed dye was released with 2% sodium dodecyl sulfate (SDS) and mixed thoroughly before transferring to a 96-well microplate and reading at 560nm using a Tecan SpectraFluor microplate reader (Tecan US, Durham, NC). Dose-response curves were generated by fitting a standard, four-parameter sigmoid curve to the data.

Western Blotting: Cancer cells were seeded in 12-well plates at concentrations of $7.5x10^4$ and $20x10^4$ cells/mL in a volume of 1mL. Cells were lysed using RIPA buffer supplemented with 1:100 protease inhibitor cocktail V (CalBioChem, USA) and collected into Eppendorf tubes using a cell scraper. The samples were sonicated for 2 seconds and centrifuged at 13000g for 10 minutes at 4ºC. The supernatant was carefully separated from the pellet into a new tube for sample preparation. Protein concentration was determined by the BCA Protein Assay Kit (Pierce, ThermoFisher Scientific). Samples were prepared and boiled for 5 minutes prior to loading. Proteins were separated on a 7.5% Tris Bis-acrylamide gel prepared the same day at 96V (E-C Apparatus Corp., EC-105) until adequate separation was achieved. Samples were transferred onto a nitrocellulose membrane at room temperature at 300mA for 1.5 hours (E-C Apparatus Corp., EC135-90). After transferring, membranes were blocked in 5% w/v non-fat dry milk in tris buffered saline with 0.5% Tween-20 (TBS-T). Membranes were probed with primary antibodies in 5% w/v non-fat dry milk overnight at 4ºC on a rotator. The primary antibodies used were rabbit anti-GAPDH (1:10000, G9545, Sigma), rabbit anti-E-cadherin (1:1000, 24E10, Cell Signaling), and rabbit anti-vimentin (1:3000, Cat# ab92547, Abcam). After primary incubation, membranes were washed 3 times for 10 minutes each in TBS-T. Anti-rabbit IgG (1:5000, Sigma, A9169) was applied at room temperature in 5% w/v non-fat dry milk for 1 hour. After secondary incubation, membranes were washed 3 times for 10 minutes each in TBS-T. Membranes were incubated with ECL western blotting substrate (Pierce, ThermoFisher Scientific) and photo developed. Western blots were scanned at a resolution of 300 DPI and grayscale bit depth of 16.

Hepatocyte – Breast Cancer Co-Culture: Primary human hepatocytes ($6x10^5$) were seeded onto Nunc Thermanox Plastic coverslips (Cat# 174950, ThermoFisher) in 12-well plates on Day 0. On Day 1, 1000 MDA-MB-231 RFP or 5000 MCF-7 RFP cells were seeded in each

well. On Day 2, cells were treated with atorvastatin or rosuvastatin, with or without LY294002 or PD98059, for 72 hours. On the last 24 hours of drug treatment, EdU (Cat# E10187, ThermoFisher) was added to a final concentration of 10μM to detect dividing cells. After 72 hours of treatment, treatment solutions were aspirated and cells were fixed with 3.7% formaldehyde (F79-1, ThermoFisher) for 15 minutes and then changed to PBS before further processing.

Microphysiological System (MPS) for Breast Cancer Metastasis in the Liver: The Liver MPS System (CNBio Innovations) was employed as previously described[1,47,750,751], and is schematized in Figure 4A. In brief, $6x10^5$ primary human hepatocytes and $6x10^5$ primary human non-parenchymal cells were seeded on high impact polystyrene scaffolds coated with 1% rat tail collagen type I (BD Biosciences) on Day 0. On Day 3, 1000 MDA-MB-231 RFP cells were seeded on top of established hepatocyte microtissues. From Day 7-10, scaffolds were treated with 1μM Doxorubicin (APP Pharmaceuticals LLC). From Day 11-15, scaffolds were treated with 5μM atorvastatin. Finally, from Day 13-15, scaffolds were treated with 1μg/mL LPS (Sigma) and 20ng/mL mouse EGF (Corning). Scaffolds were continuously perfused at a rate of 1μL/s over the entire culture duration. Media were changed every two days and the supernatant was collected for analysis by the University of Pittsburgh Medical Center CLIA-certified clinical labs. On Day 15, the scaffolds were fixed with 2% paraformaldehyde in PBS (Sigma) for 1 hour at 4ºC and then changed to PBS before further processing.

Mice and Drug Treatments: NOD-SCID gamma (NSG) female mice were obtained from Jackson Labs. Mice were 8 weeks of age upon the start of all experiments. Mice were injected intraperitoneally (IP) with atorvastatin at 2mg/kg or 10mg/kg, EdU at 10mg/kg, or the 2% DMSO vehicle at a volume of 10μL/mg body weight. Mice were euthanized by asphyxiation in a chamber with adequate CO_2 flow as per AVMA procedures.

Intrasplenic Inoculation Model for Breast Cancer Metastasis to the Liver: On Day 0, mice were anesthetized, their spleens were exposed using a left lateral abdominal incision, and $5x10^4$ MDA-MB-231 RFP cells were injected into the spleen in a volume of 50μL using a 26.5 gauge needle. The peritoneum was closed using one simple stitch with a 5-0 vicryl suture and the skin was closed using staples. Mice were kept on a heating blanket and monitored until they recovered from surgery and were subsequently housed individually. On Day 7, daily IP injections of atorvastatin (at 2mg/kg or 10mg/kg) or vehicle were started. On Day 26, two days before euthanizing the mice, a single EdU IP injection was given. On Day 28, mice were euthanized and the spleen, liver, and quad muscle were harvested for analysis.

Mammary Fat Pad Inoculation Model for Breast Cancer Metastasis to the Lung: On Day 0, $1x10^6$ MDA-MB-231 RFP cells were injected into the right inguinal mammary fat pad in a volume of 200μL using a 26.5 gauge needle and were subsequently housed in cages of two. On Day 10, six IP injections per week of atorvastatin (at 2mg/kg or 10mg/kg) or vehicle were started. On Day 30, mice were euthanized and the mammary tumor and lungs were harvested for analysis.

Tissue Histology: Harvested mouse tissues were immediately fixed in 10% neutral buffered formalin and submitted to the University of Pittsburgh Health Sciences Tissue Bank, where the tissue was paraffin embedded and sectioned. Additionally, Ki-67 and E-cadherin immunohistochemistry (IHC), terminal deoxynucleodityl transferase dUTP nick end labeling (TUNEL), and hematoxylin and eosin (H&E) staining were performed by their well validated protocols[921]. Blank splenic tumor and liver tumor slides were obtained for in house EdU staining.

E-cadherin and Vimentin Immunofluorescence: MCF-7 RFP, MDA-MB-231 RFP, and MDA-MB-231 RFP/Ecad cells were fixed and probed using mouse anti-E-cadherin (1:500, Cat# 13-5700, Invitrogen) or rabbit anti-vimentin (1:200, Cat# ab92547, Abcam) overnight at 4^{o}C. The

appropriate species secondary antibody was used - goat anti-mouse Alexa Fluor 488 (1:200, Cat# A-11001, ThermoFisher) or goat anti-rabbit Alexa Fluor 488 (1:200, Cat# A-11008, ThermoFisher) - for 1 hour at room temperature. After antibody staining, cells were counterstained with 2.5µg/mL DAPI for 15 minutes at room temperature and washed three times in normal saline. Coverslips were mounted using a glycerol and PVA based mounting medium, courtesy of the Center for Biological Imaging at the University of Pittsburgh, and were allowed to harden overnight prior to imaging.

EdU Staining: Cells fixed on coverslips, MPS scaffolds, or mouse tissue slides were permeabilized with 0.1% Triton-X-100 (ThermoFisher) for 20 minutes then washed once with 3% Bovine Serum Albumin (Sigma). Cells were stained for EdU using the Click-iT EdU Alexa Fluor 488 Imaging Kit (ThermoFisher) per the manufacturer's instructions. After EdU staining, the cells were counter-stained with 2.5µg/mL (coverslips) or 10µg/mL (MPS scaffolds) DAPI for 15 minutes at room temperature, and washed three times in normal saline. Coverslips were mounted using a glycerol and PVA based mounting medium, courtesy of the Center for Biological Imaging at the University of Pittsburgh, and were allowed to harden overnight prior to imaging. MPS scaffolds were kept in PBS until imaging.

Imaging: E-cadherin and vimentin stained cells (Figure 46) and MPS scaffolds (Figure 49) were imaged on a Nikon A1 confocal microscope (Nikon) using 40x and 10x objectives (Olympus), respectively. For MPS scaffolds, the large image and Z-stack features of the A1 scope were used, to take 4x4 fields of view for each of seven Z-planes, spaced 20µm apart. The images shown are the maximum intensity projection of the z-stacks whereas the quantification was performed by summation of the individual stacks. For the hepatocyte – breast cancer co-culture coverslips (Figure 47, Figure 48, Figure 55), cells were imaged on a 90i widefield microscope

(Nikon) using a 10x objective (Olympus). The large image feature was used to take 10x14 fields of view (60mm^2, 45% coverslip area) for each coverslip. Use of these two scopes was generously provided by the Center for Biological Imaging at the University of Pittsburgh, supported by NIH grant 1S10OD019973-01. Mouse tissue images (Figures 50-53, Figure 57, Figure 58) were taken using an Olympus BX40 upright microscope with a 10x and 40x objective using either brightfield (H&E, TUNEL, and Ki-67 IHC images) or fluorescence excitation wavelengths of 405nm (DAPI) and 488nm (Click-iT EdU).

Image Analysis: Image analysis was performed in NIS Elements version 5.0. Nuclei (DAPI) and proliferating nuclei (EdU) channels were labeled using spot detection for bright, clustered spots, and using the same parameters for all images associated with that experiment. The thresholding function was used to label all RFP-positive area (RFP), which represents the area encompassed by the breast cancer cells. Once individual channel masks were created, combined channel masks were generated by using the "having" command, which creates a new mask that illustrates all pixels of the first mask that contain at least one pixel of the second. After generating all masks, data were measured and extracted for analysis. Specifically, the parameters reported are: Nuclei Number (DAPI), % Proliferating Cancer Cells ((DAPI + RFP + EdU) / (DAPI + RFP)), and Normalized Area (percent area of the total image encompassed by the RFP threshold, normalized to the vehicle control).

Statistics: In all figures, the data are presented as the mean of three independent experiments, when counting the experiments were performed in triplicate, with the error bars representing the standard error of the mean, unless otherwise stated. Comparisons of individual columns in Figure 47 and Figure 49 were determined by use of a student's two-tailed unequal variance t-test. Comparisons of dose curves in Figure 48 were made using a two way ANOVA

without sample matching, with significance reported as the probability for interaction between the atorvastatin and LY294002 or PD98059 treatment, to test for potentiation. Comparisons in Figures 50-54 were performed using Kruskal Wallis one way ANOVA, with post-hoc Dunn's multiple comparison test to determine significance between individual columns. Significance levels are reported in the figure legends and are kept consistent across all figures with symbols denoting * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

8.3 PRIMARY DATA

8.3.1 Mesenchymal breast cancer cells that lack membrane E-cadherin are more sensitive to atorvastatin suppression

We previously demonstrated that membrane E-cadherin was a marker and mechanism of resistance to atorvastatin treatment, whereas vimentin did not correlate with atorvastatin resistance[2]. Herein in breast cancer cells, we found the mesenchymal MDA-MB-231 cells were more sensitive to atorvastatin-mediated growth suppression than the epithelial MCF-7 cells (Figure 46A). As we demonstrated previously[2], expression of membrane E-cadherin on MDA-MB-231 cells was sufficient to increase resistance to atorvastatin treatment, though not to the same level as MCF-7 (Figure 46). To compare epithelial and mesenchymal nature of these cell lines, we determined expression and localization of E-cadherin and vimentin by western blot (Figure 46B) and immunofluorescent microscopy (Figure 46C-H). MCF-7 cells expressed E-cadherin to a greater level and with more membrane presentation than MDA-MB-231 Ecad while MDA-MB-231 cells did not express detectable E-cadherin (Figure 46B-E). In contrast, MCF-7 cells did not express

detectable vimentin whereas MDA-MB-231 and MDA-MB-231 Ecad cells expressed vimentin that stained in a filamentous cytoplasmic pattern (Figure 46B,F-H). These findings confirm our earlier reports[2,3] of E-cadherin membrane expression correlating with relative resistance to statin growth suppression.

8.3.2 Atorvastatin suppresses growth of mesenchymal breast cancer cells in co-culture with primary human hepatocytes

The liver is a common site for breast cancer metastasis and is the primary site for drug metabolism in the body[962]. As such, we next wanted to determine whether hepatocytes would influence the efficacy of atorvastatin against breast cancer cells. To accomplish this goal, we co-cultured primary human hepatocytes with MCF-7 RFP or MDA-MB-231 cells for 4 days (Figure 47A). Importantly, less than 1% of the cells in these co-cultures were breast cancer cells, in order to model the relative overabundance of liver resident cells as compared to tumor cells in the context of micrometastases[750]. After allowing the breast cancer cells to integrate into the established hepatocyte monolayer, we treated the co-cultures with atorvastatin for 72 hours and included EdU in the culture media for the last 24 hours to detect any cells that transited the S phase (Figure 47A). After treatment, the co-cultures were fixed, imaged using widefield microscopy, and quantified by image analyses to assess statin toxicity (total nuclei number), influence on breast cancer proliferation (% EdU+ cancer cells), and tumor burden (normalized tumor area).

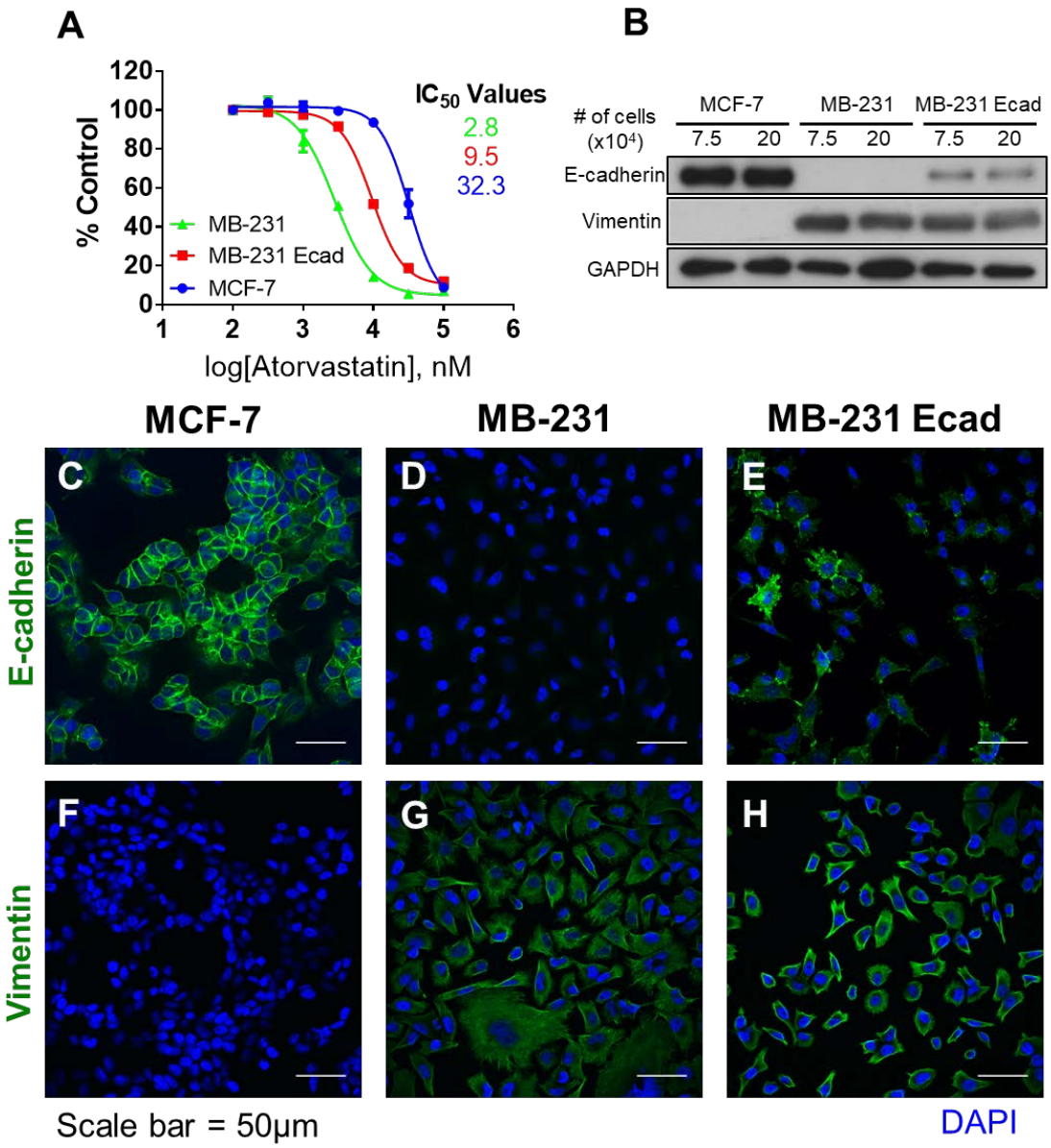


Figure 46. Atorvastatin suppresses mesenchymal breast cancer cells

(A) MCF-7 RFP, MDA-MB-231 RFP, and MDA-MB-231 RFP/Ecad were grown in the presence of atorvastatin for 72 hours and total cell number was determined by crystal violet staining and normalized to the control. IC_{50} values for each cell line are reported. (B) MCF-7 RFP, MDA-MB-231 RFP, and MDA-MB-231 RFP/Ecad were seeded either at $7.5x10^4$ or $20x10^4$ cells per well and probed for their total cell levels of E-cadherin and vimentin. The expression of (C-E) E-cadherin and (F-H) vimentin in (C,F) MCF-7 RFP, (D,G) MDA-MB-231 RFP, and (E,H) MDA-MB-231 RFP/Ecad was probed by immunofluorescent imaging. The data are representative of three independent experiments. Scalebar = 50μm.

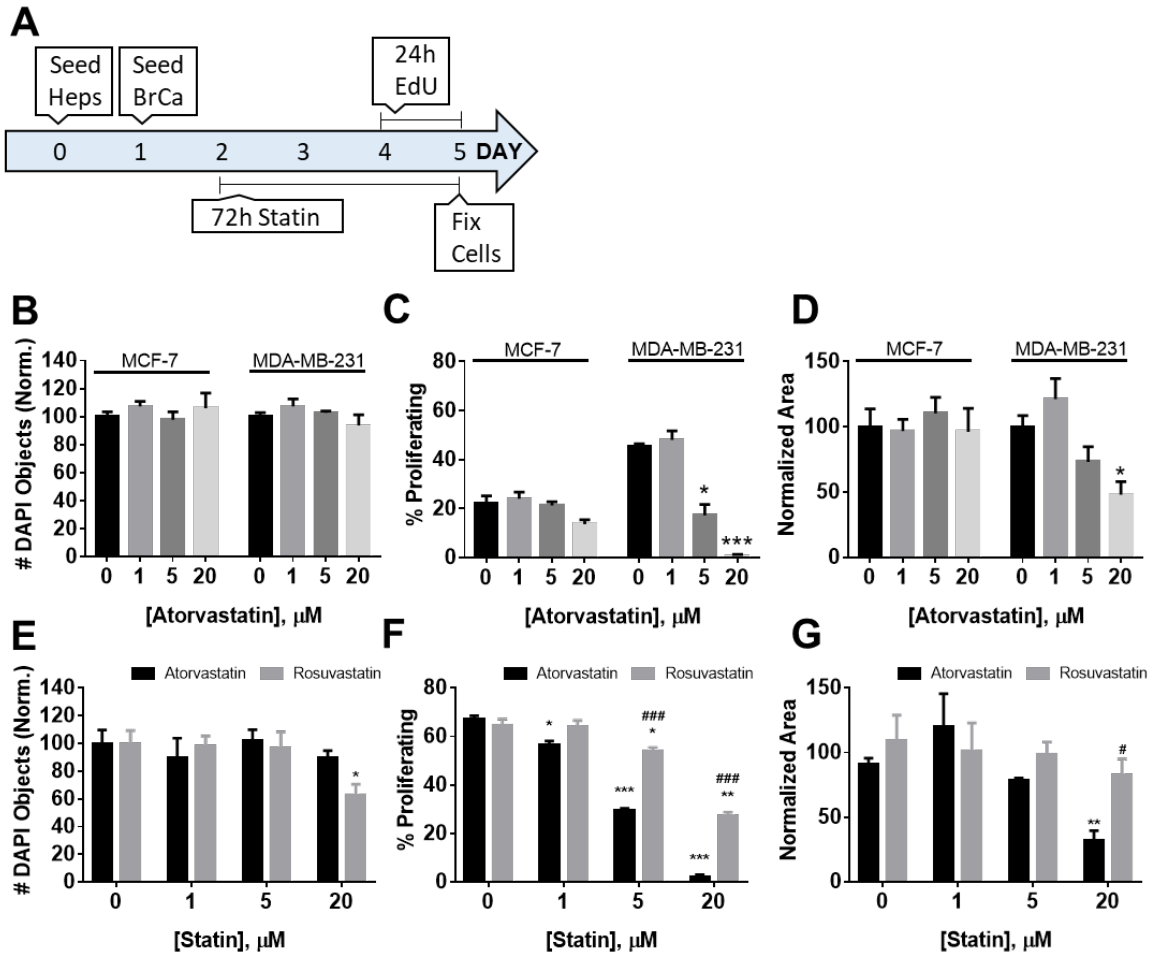


Figure 47. Atorvastatin is more effective than rosuvastatin in 2D co-culture with hepatocytes

(A) The experimental outline for hepatocyte – breast cancer co-culture using primary human hepatocytes (Heps; $6x10^5$) and RFP tagged breast cancer cells (BrCa; 5000 MCF-7 RFP or 1000 MDA-MB-231 RFP). (B-D) Atorvastatin treatment in co-cultures with MCF-7 RFP or MDA-MB-231 RFP. (B) Atorvastatin did not influence total nuclei number, but did reduce MDA-MB-231 RFP (C) proliferation and (D) total cancer cell area. (E-G) Atorvastatin and rosuvastatin treatment in hepatocyte – MDA-MB-231 RFP co-cultures. (E) Rosuvastatin demonstrated a reduction in nuclei number at the highest dose and a lesser potency at (F) suppressing proliferation and (G) total cancer cell area than atorvastatin. The data are representative of three independent experiments. Error bars represent the standard error of the mean (n=3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to vehicle. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to the same dose of atorvastatin.

Atorvastatin treatment did not affect the total nuclei number (Figure 47B). While atorvastatin treatment significantly decreased MDA-MB-231 proliferation in a dose-dependent manner, proliferation of MCF-7 cells was unaffected (Figure 47C). Similarly, atorvastatin decreased tumor burden in co-cultures with MDA-MB-231 cells but not those with MCF-7 cells (Figure 47D). Atorvastatin appears to be less potent at suppressing growth in this 2D co-culture system than in 2D monoculture of breast cancer cells (Figure 46).

We previously demonstrated that the lipophilic atorvastatin was more potent at suppressing cancer cell growth than the hydrophilic rosuvastatin in cancer cell line monolayer cultures[3]. To determine whether this effect persisted in the context of the hepatic microenvironment, we co-cultured MDA-MB-231 cells with primary human hepatocytes. In contrast to atorvastatin treatment, we found that rosuvastatin decreased total nuclei number by approximately 40% at the highest dose (Figure 47E) suggesting toxicity towards the liver cells. The hepatotoxicity of rosuvastatin has been reported in the literature and is thought to be due to the higher selective uptake of rosuvastatin by hepatocytes as compared to other statins[963]. Rosuvastatin was significantly less potent than atorvastatin at all tested doses at suppressing the growth of the MDA-MB-231 cells (Figure 47F). Thus, while the highest dose of atorvastatin was able to significantly suppress tumor burden, the same dose of rosuvastatin was ineffective (Figure 47G).

Even though rosuvastatin has a higher *in vitro* affinity for its target enzyme, HMGCR, than atorvastatin[562], it exhibit lower potency at suppressing MDA-MB-231 proliferation. While the lipophilic atorvastatin can diffuse across tumor cell membranes, the hydrophilic rosuvastatin relies more on active transport. Hepatocytes but not breast cancer cells express the OATP1B1 and 1B3 transporters responsible for statin cellular uptake[592]. We have previously shown that atorvastatin is more effective than rosuvastatin at suppressing cancer cell growth[3,4]. These data additionally

suggest that the direct effects of statins on tumor cells play a larger role in proliferation suppression than indirect effects from statins influencing the neighboring hepatocytes. Moreover, we found atorvastatin could reduce tumor burden – defined as the normalized area of breast cancer cells - at the highest dose whereas rosuvastatin was ineffective (Figure 47). These data also suggest that, in the context of the hepatic microenvironment, statins act mainly in a cytostatic manner, as the anti-proliferation effects occur at lower doses than a reduction in tumor burden (Figure 47). In support, previous studies have shown statins can induce apoptosis in breast cancer cells *in vitro* but not in human biopsy samples after neoadjuvant statin treatment in breast cancer patients[699,706].

8.3.3 PI3K inhibition potentiates atorvastatin-mediated suppression of MDA-MB-231 proliferation in co-culture with primary human hepatocytes

The PI3K-Akt and MAP kinase signaling pathways play significant roles in breast cancer cell growth and survival[200,237]. To determine whether these pathways played a role in breast cancer sensitivity to atorvastatin, we co-cultured MDA-MB-231 cells with primary human hepatocytes (Figure 48A). During atorvastatin treatment, cells were additionally treated with 3μM or 10μM LY294002 (a pan-PI3K inhibitor) or 10μM PD98059 (a Mek1/2 inhibitor). Inhibition of PI3K or Mek1/2 did not significantly influence hepatocyte health (Figure 48B) or tumor burden (Figure 48C) as compared to atorvastatin treatment alone, but PI3K inhibition potentiated atorvastatin-mediated suppression of MDA-MB-231 proliferation in a dose dependent manner (p-value = 0.01) (Figure 48D,E). In contrast, Mek1/2 inhibition had no effect on potentiating atorvastatin (Figure 48D,E). These results are consistent with our finding that, in breast cancer cell lines, atorvastatin decreases Ras prenylation, its attachment to intracellular membranes, and thus activation[4].

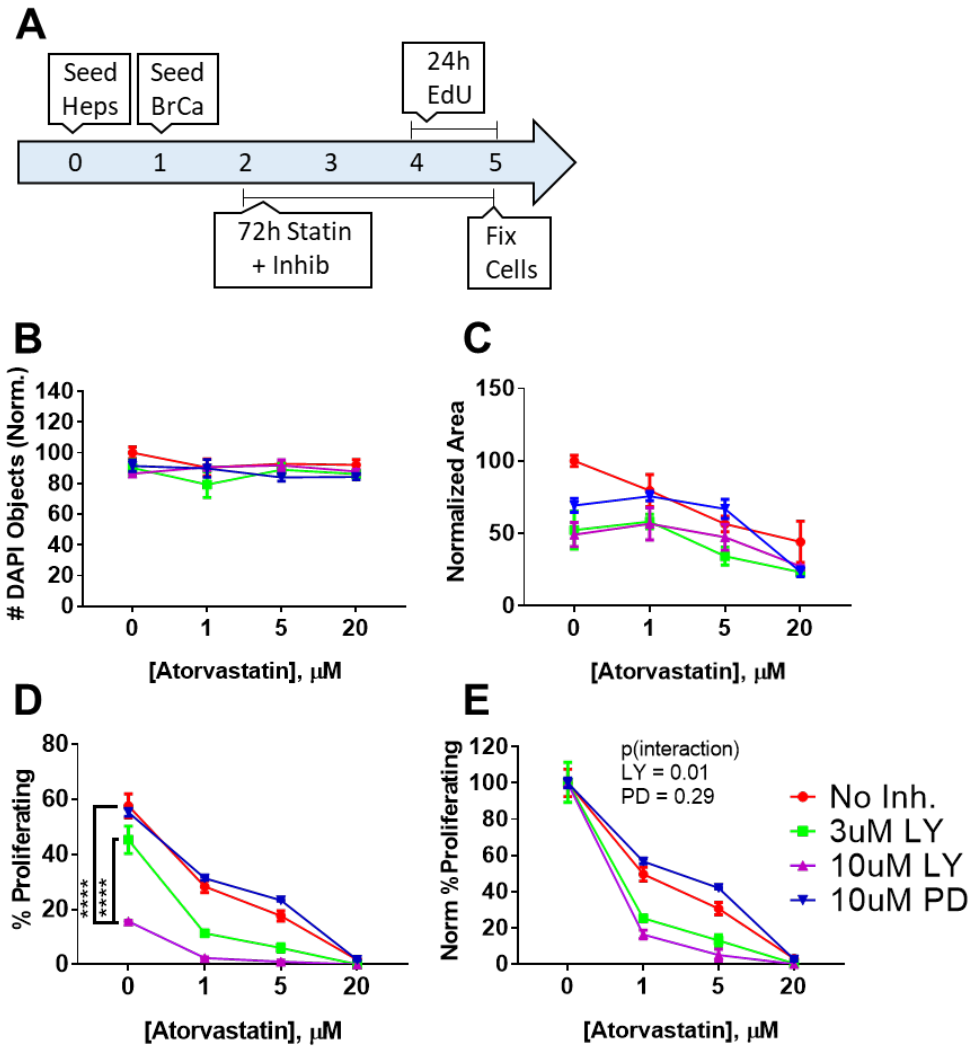


Figure 48. PI3K inhibition potentiates atorvastatin in 2D co-culture with hepatocytes

(A) The experimental outline for hepatocyte – breast cancer co-culture using primary human hepatocytes (Heps; 6x105) and RFP tagged breast cancer cells (BrCa; 1000 MDA-MB-231 RFP). Co-cultures were treated with atorvastatin for 72 hours with 3μM or 10μM LY294002 (LY; PI3K inhibitor), 10μM PD98059 (PD; Mek1/2 inhibitor), or vehicle. (B) Nuclei number and (C) cancer cell area were unaffected by PI3K or Mek1/2 inhibition. PI3K inhibition but not Mek1/2 inhibition significantly inhibited MDA-MB-231 RFP (D) proliferation and (E) potentiated the growth suppressive effect of atorvastatin, illustrated by the p-value for interaction by two-way ANOVA. The data are representative of three independent experiments. Error bars represent the standard error of the mean (n=3). The data are representative of three independent experiments. **** $p < 0.0001$.

8.3.4 Atorvastatin suppresses stimulated outgrowth dormant breast cancer cells

Since we found atorvastatin could decrease the proliferation of breast cancer cells, we next wished to determine whether atorvastatin could suppress stimulated outgrowth of dormant breast cancer cells in the context of the liver microenvironment. To accomplish this goal, we employed an all-human microphysiological system (MPS) for breast cancer metastasis in the liver. This system has been extensively characterized and has been shown to model metastatic dormant-emergent breast cancer[47,750,898]. In this system, a micro-hepatic tissue is established by seeding primary human liver cells, hepatocytes and a full complement of non-parenchymal cells, in a porous scaffold subject to a physiological flow. A full description of the functionality of the MPS has been published[753,964]. In brief, RFP-labeled breast cancer cells are seeded into these microtissues, perturbed by various drug treatments, and examined two weeks later by clinical chemistry assays and microscopy.

The experimental outline is shown in Figure 49A. In summary, we first treated cells with doxorubicin for 72 hours to eliminate cycling cells so that the dormant cell fraction remained. We next treated with atorvastatin for 4 days and on the last two days of treatment, stimulated with an LPS/EGF cocktail, a physiologically relevant inflammatory stimulus designed to drive dormant breast cancer cells to emerge and proliferate[750]. After two days of stimulation, we fixed the cells and imaged scaffolds on a confocal microscope (Figure 49B-E). Atorvastatin significantly suppressed LPS/EGF stimulated outgrowth of the mesenchymal MDA-MB-231 cells (Figure 49F). These data suggest that atorvastatin can suppress emergence of the statin sensitive MDA-MB-231 cells from tumor dormancy. Similar to in 2D co-culture (Figure 47), statins appear to act as cytostatic agents, as the normalized cancer area is similar between doxorubicin treated scaffolds and doxorubicin, atorvastatin, and LPS/EGF treated scaffolds (Figure 49F).

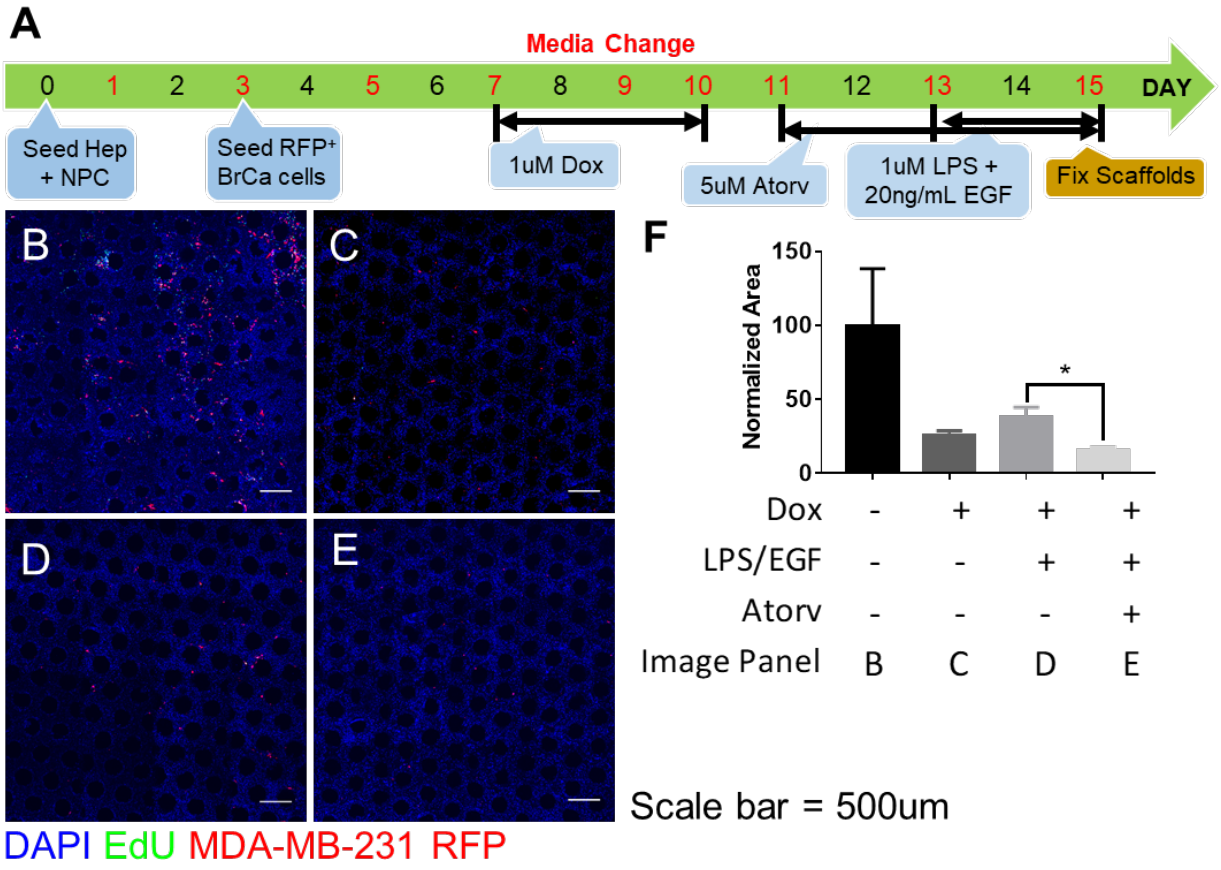


Figure 49. Atorvastatin suppresses stimulated outgrowth of mesenchymal breast cancer cells

(A) The experimental outline for microphysiological system culture of primary human hepatocytes (Hep; 6x105), non-parenchymal cells (NPC; 6x105), and MDA-MB-231 RFP cells (1000), with media changes represented as red day numbers. Confocal microscopy was used to generate large Z-stack images for analysis, with representative images shown for each drug treatment: (B) vehicle, (C) Dox, (D) Dox + LPS/EGF, and (E) Dox + LPS/EGF + Atorv. Red = RFP, Green = EdU, Blue = DAPI. (F) Atorvastatin treatment was able to suppress LPS/EGF stimulated outgrowth of MDA-MB-231 RFP cells. Dox = 1µM doxorubicin, LPS/EGF = 1µg/mL LPS + 20ng/mL EGF, and Atorv = 5µM atorvastatin. The data are representative of two independent experiments each of which with a technical n = 2. Error bars represent the standard error of the mean (n=4). * $p < 0.05$. Scale bar = 500µm.

8.3.5 Atorvastatin suppresses proliferation of breast cancer liver metastases but not the primary tumor

Since we found atorvastatin was able to suppress the stimulated outgrowth of breast cancer cells in the liver MPS, we wanted to examine whether systemic atorvastatin administration in a mouse model for spontaneous breast cancer metastasis to the liver would show the same affect. The experimental outline is shown in Figure 50A. In summary, we inoculated MDA-MB-231 into the spleens of female NSG mice. After allowing the primary tumor to establish for 1 week, we treated the mice with daily IP injections of 2mg/kg atorvastatin, 10mg/kg atorvastatin, or vehicle for 3 weeks. Two days before euthanizing the mice, we also injected 10mg/kg EdU to detect cell proliferation. We found that all mice developed primary tumors in the spleen without having overt liver macrometastases. To assess statin toxicity we examined skeletal muscle; all mice exhibited healthy quadriceps (quad) muscle tissue (Figure 50B).

We found the primary tumor size did not significantly change with atorvastatin treatment (Figure 5D). Primary tumors grew alongside the splenic parenchyma and would often invade through the splenic capsule as they grew (Figure 50C). Primary tumor proliferation was quantified by assessing the EdU positivity of the peripheral zone of the tumor. The proliferation of the primary tumor was not significantly affected by atorvastatin treatment (Figure 50E,F).

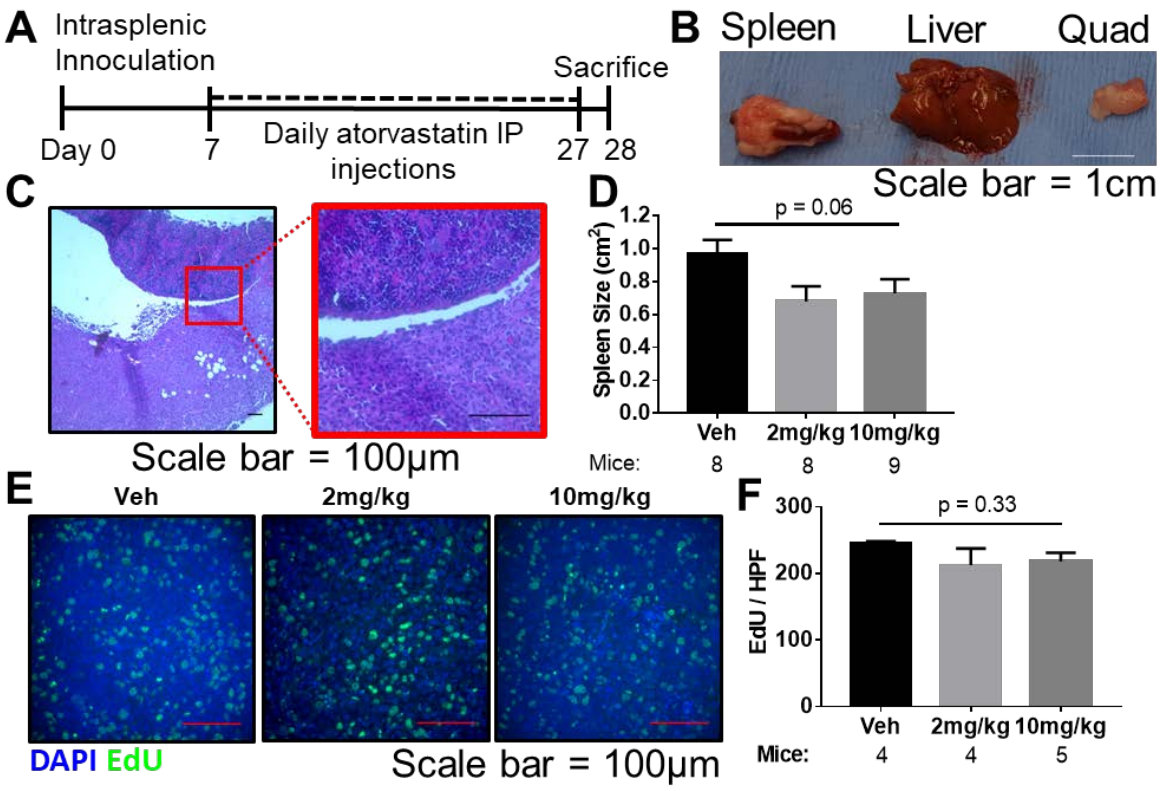


Figure 50. Atorvastatin does not suppress breast cancer primary splenic tumor growth

(A) The experimental outline for a splenic inoculation model for spontaneous breast cancer metastasis to the liver. MDA-MB-231 RFP cells ($5x10^4$) were inoculated into the spleens of mice on day 0, mice were treated with daily intraperitoneal (IP) injections of 2mg/kg or 10mg/kg atorvastatin or vehicle (Veh, 2% DMSO in saline) starting on day 7, and mice were euthanized on day 28 for tissue harvesting. (B) Gross pathology was used to examine primary tumor size, the presence or lack of overt liver macrometastases, and myotoxicity. (C) Histology was determined by H&E staining and (D) primary tumor size was determined by splenic cross-sectional area. (E,F) Primary tumor proliferation was assessed by EdU incorporation on the two days prior to harvesting tissue, with EdU represented in green and DAPI counterstaining shown in blue. Error bars represent the standard error of the mean. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. White scale bar = 1cm, red and black scale bars = 100µm.

We found that the total size of the liver did not significantly change with atorvastatin treatment (Figure 51A). Breast cancer metastases ranged in size, but were at the largest several hundred micrometers in diameter (Figure 51B); these nodules still represented only a minor fraction of total liver tissue reflecting the micrometastatic nature of the tumor nodules. To assess proliferation of liver metastases, EdU staining was performed. Tumor nodules were easy to identify by the lack of green hepatocyte auto-fluorescence (Figure 51C). We found that atorvastatin significantly decreased proliferation density of metastatic nodules in a dose dependent manner (Figure 51D).

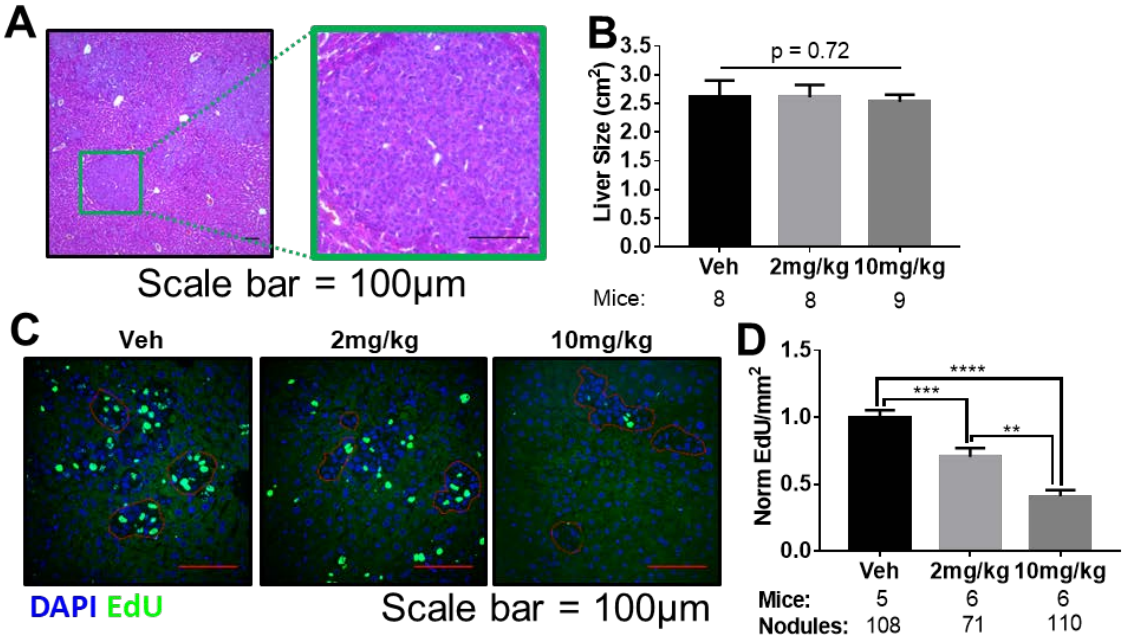


Figure 51. Atorvastatin suppresses breast cancer liver metastatic proliferation

The paired liver metastasis data with the splenic primary data shown in Figure 50. (A) The histology of liver metastases was determined by H&E staining and (B) gross metastatic burden was assessed by liver cross-sectional area. (C,D) Liver metastases proliferation was assessed by EdU incorporation on the two days prior to harvesting

tissue, with EdU represented in green and DAPI counterstaining shown in blue. Error bars represent the standard error of the mean. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Red and black scale bars = 100µm.

8.3.6 Atorvastatin suppresses proliferation of breast cancer lung metastases but not the primary tumor

Since we found atorvastatin could significantly suppress the growth of micrometastases, we next wanted to determine if atorvastatin would reduce metastatic proliferation at a different site: the lung, the most common site for clinically evident breast cancer metastasis[50]. The experimental outline is shown in Figure 52A. In summary, we inoculated MDA-MB-231 breast cancer cells into the inguinal mammary fat pad (MFP) of female NSG mice. After allowing the primary tumor to establish for 10 days, we treated the mice with six IP injections per week of 2mg/kg or 10mg/kg atorvastatin or vehicle for 3 weeks. We found that all mice developed primary tumors and were void of overt lung macrometastases (Figure 52B).

We found the primary tumor size did not significantly change with atorvastatin treatment (Figure 52D). Primary tumors were marked by central regions of necrosis (Figure 52C). Primary tumor proliferation was quantified by assessing the Ki-67 positivity of the peripheral zone of the tumor. The proliferation of the primary tumor was not significantly affected by atorvastatin treatment (Figure 52E,F). In contrast to the primary tumor, the lung metastases were much smaller and exhibited no necrotic regions (Figure 53A). Moreover, we found that atorvastatin reduced the proliferation of lung metastases in a dose dependent manner, though this trend did not reach statistical significance in the small number of animals challenged (Figure 53H,I).

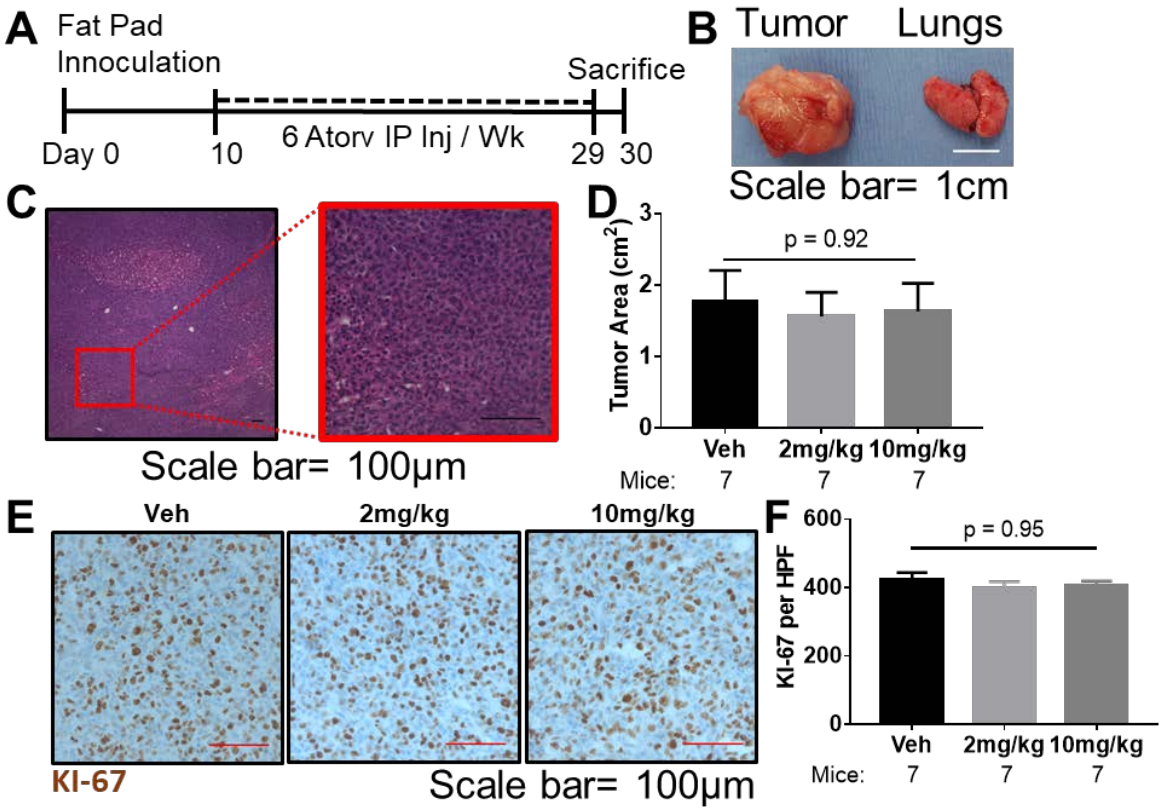


Figure 52. Atorvastatin does not suppress breast cancer primary fat pad tumor growth

(A) The experimental outline for a mammary fat pad (MFP) inoculation model for spontaneous breast cancer metastasis to the lung. MDA-MB-231 RFP cells (5x105) were inoculated into the right MFP of mice on day 0, mice were treated with six per week intraperitoneal (IP) injections of 2mg/kg or 10mg/kg atorvastatin or vehicle (Veh, 2% DMSO in saline) starting on day 10, and mice were euthanized on day 28 for tissue harvesting. (B) Gross pathology was used to examine primary tumor size and the presence or lack of overt lung macrometastases. (C) Primary tumor histology was determined by H&E staining and (D) size was determined by tumor cross-sectional area. (E,F) Primary tumor proliferation was assessed by Ki-67 immunohistochemistry, with Ki-67 represented in brown and counterstained by hematoxolin. Error bars represent the standard error of the mean. White scale bar = 1cm, red and black scale bars = 100µm.

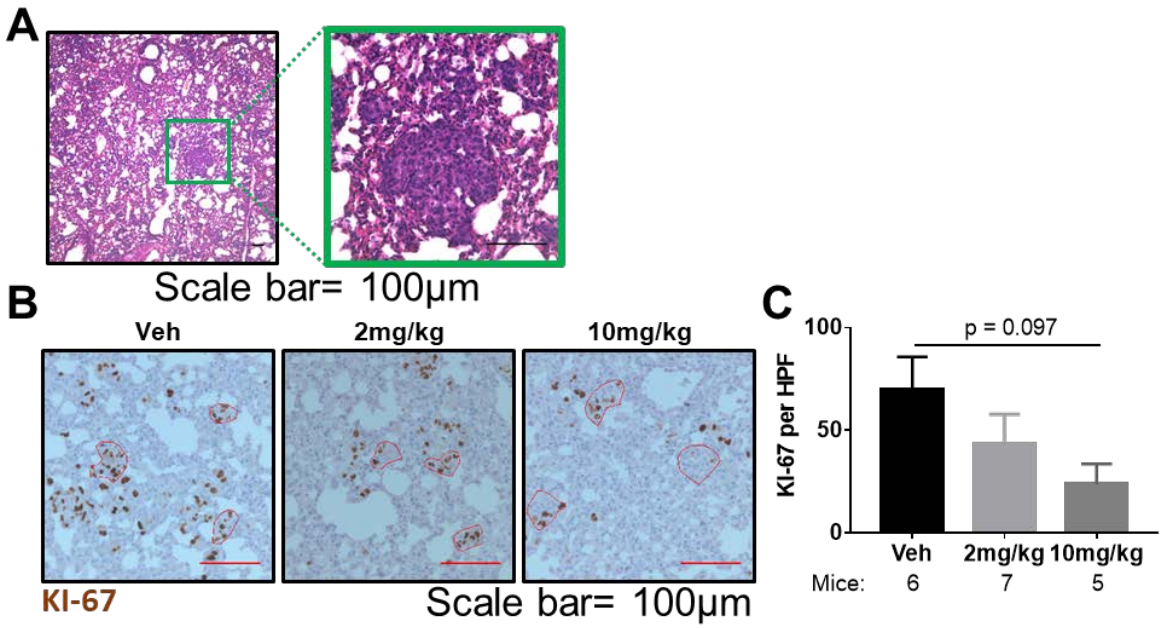


Figure 53. Atorvastatin suppresses breast cancer lung metastatic proliferation

The paired lung metastasis data with the fat pad primary data shown in Figure 52. (A) Histology of lung metastases was determined by H&E staining. (B,C) Lung metastases proliferation was assessed by Ki-67 immunohistochemistry, with Ki-67 represented in brown and counterstained by hematoxolin. Examples of individual tumor nodules are circled in red. Significant outliers (n=1 in the vehicle and 10mg/kg atorvastatin groups) as detected by Grubbs' test were removed from the analysis in I. Error bars represent the standard error of the mean. Red and black scale bars = 100µm.

8.3.7 Atorvastatin suppresses growth of metastases but not the primary tumor

As demonstrated in the previous two subsections, we employed two independent models of spontaneous breast cancer metastasis: spleen-to-liver and mammary fat pad (MFP)-to-lung. Importantly, these models encompass the entire metastatic cascade, beginning with growth of a primary tumor and ending with distant metastases. This is in contrast to tail vein or intra-cardiac injection models, which primarily probe direct hematogenous seeding of cancer cells and bypass the pathophysiologic changes needed for both initial escape from the primary tumor and seeding as singular cells in the ectopic tissues. In our spleen-to-liver metastasis model, we found that atorvastatin treatment could significantly suppress proliferation of liver metastatic nodules but not the primary tumor (Figure 50, Figure 51). Moreover, this effect on proliferation was dose dependent. Atorvastatin did not induce cell death in either the primary tumor or metastatic nodules, confirming our *in vitro* evidence that statins mainly act as growth suppressive in the context of the tumor microenvironment (Figure 47, Figure 49). In our MFP-to-lung metastasis model, we found that atorvastatin did not affect primary tumor proliferation but reduced proliferation of lung metastases (Figure 52, Figure 53). We believe the atorvastatin-mediated suppression of lung metastasis proliferation did not achieve statistical significance due to a higher variability in spontaneous metastasis to the lung, which can be observed in vehicle treated mice.

To assess the effect of atorvastatin on any site metastasis or primary tumor, we normalized proliferation rates for both the spleen-to-liver (Figure 50, Figure 51) and MFP-to-lung (Figure 52, Figure 53) models to their respective vehicle treatments. We found that atorvastatin did not affect the proliferation of the primary tumors (Figure 54A) yet significantly decreased the proliferation of the metastases in a dose-dependent manner (Figure 54B). These data suggest that statins act to reduce metastatic tumor growth (Figure 12).

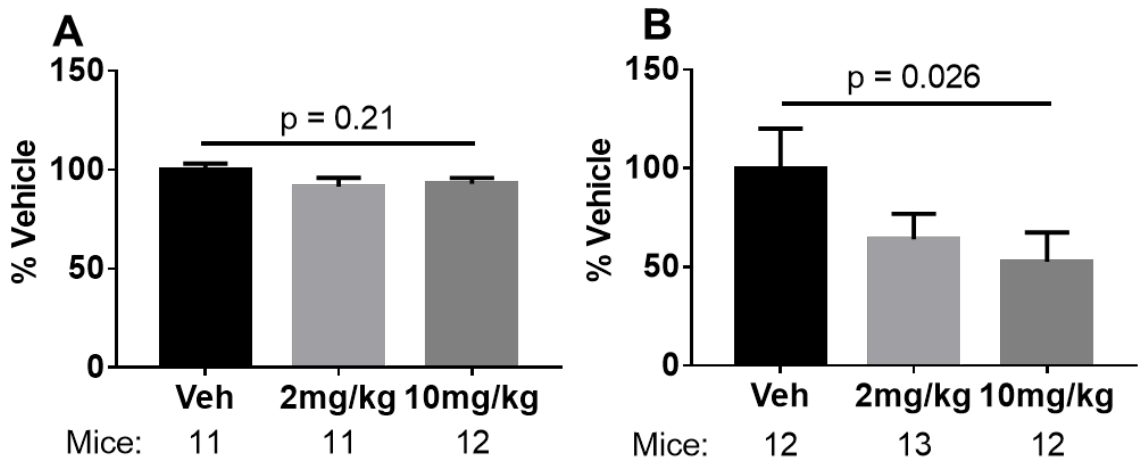


Figure 54. Atorvastatin suppresses proliferation of metastatic but not primary tumor cells

Proliferation of primary tumor cells and metastatic tumor cells obtained for the intrasplenic inoculation model of spontaneous breast cancer metastasis to liver (Figure 5) and the mammary fat pad (MFP) inoculation model of spontaneous breast cancer metastasis to lung (Figure 6) were normalized to their respective vehicle controls. (A) Primary tumor cell proliferation was assessed by combining the normalized proliferation values for the splenic and MFP experiments. (B) Metastatic tumor cell proliferation was assessed by combining the normalized proliferation values for the liver and lung metastases. Error bars represent the standard error of the mean.

8.4 SUPPLEMENTAL DATA

In order to best characterize the influence of atorvastatin and rosuvastatin treatment on 2D co-cultures of breast cancer cells and primary human hepatocytes, a high content wide-field imaging strategy was employed. For each treatment condition, 140 images were taken and stitched into one large image (Figure 55A), upon which the subsequent image analysis was performed.

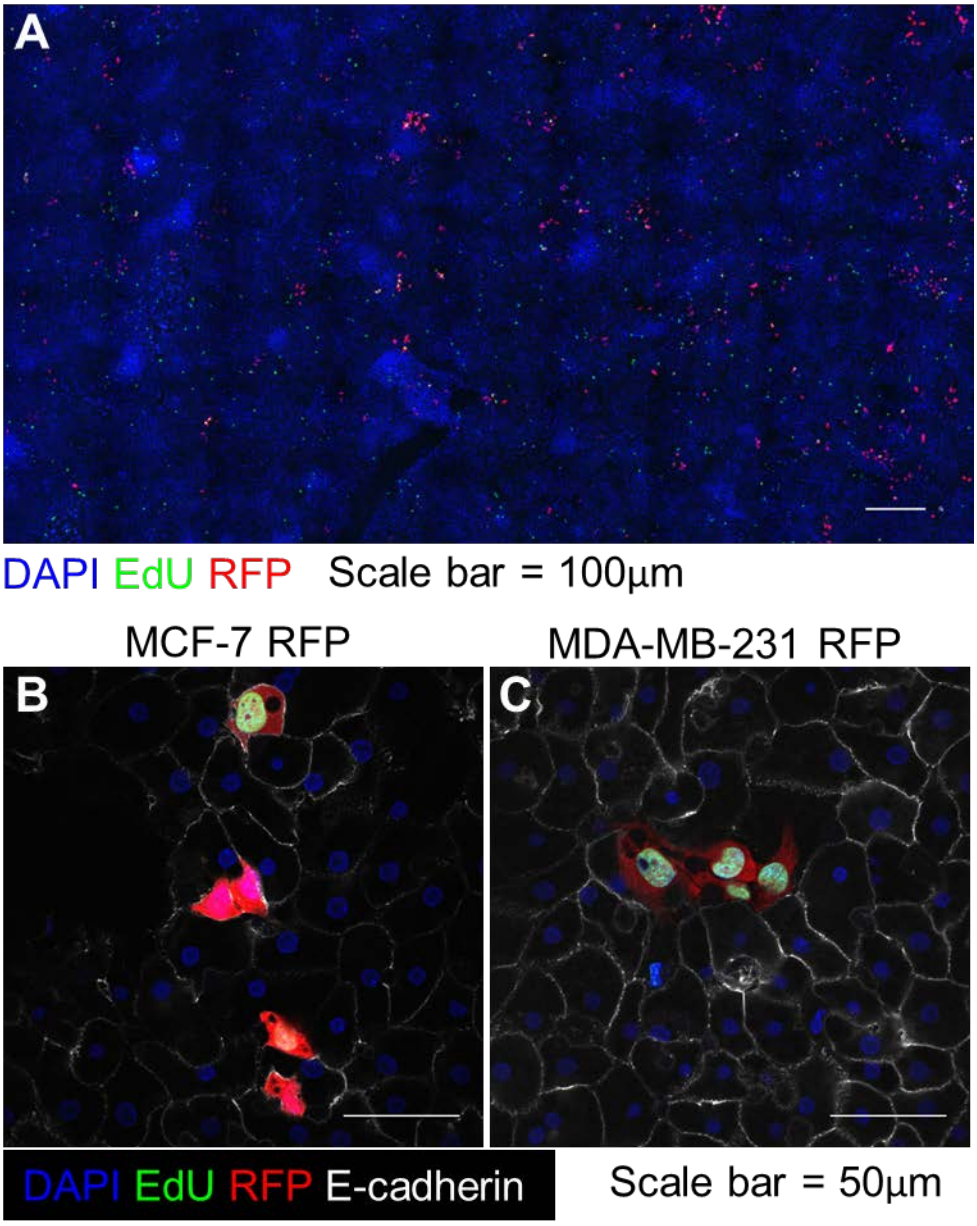


Figure 55. Breast cancer and hepatocyte co-cultures

MDA-MB-231 RFP cells (1000) and MCF-7 RFP (5000) cells were cultured with 6x105 primary human hepatocytes and fixed after 96 hours of co-culture. (A) A widefield microscope was used to take and stitch 140 congruent fields of view. Scale bar = 100µm. (B) MCF-7 RFP and (C) MDA-MB-231 RFP co-cultures were additionally stained for E-cadherin, to demonstrate cancer cell integration into the hepatocyte monolayer. Scale bar = 50µm. Blue = DAPI, Green = EdU, Red = RFP, White = E-cadherin.

Analysis of a large culture area was needed to accurately assess the influence of statin treatment due to the high variability in individual images taken (Figure 55A). Higher power imaging demonstrated that both MCF-7 (Figure 55B) and MDA-MB-231 (Figure 55C) cells could integrate into the hepatocyte monolayer. Thus, the influence of statins on the cancer cells in this system were either direct effects on the tumor cells or indirect effects through affecting the hepatocytes.

In order to determine whether atorvastatin reduced viability of hepatocytes in the liver MPS (Figure 49), clinical chemistry assays were done to assess AST and ALT release into the effluent. Similar to the results for the 2D co-culture model, in which no decrease in nuclei number was noted with atorvastatin treatment, we found none of the drug treatments damaged the hepatocytes as shown by no increase in AST and ALT release into the effluent (Figure 56).

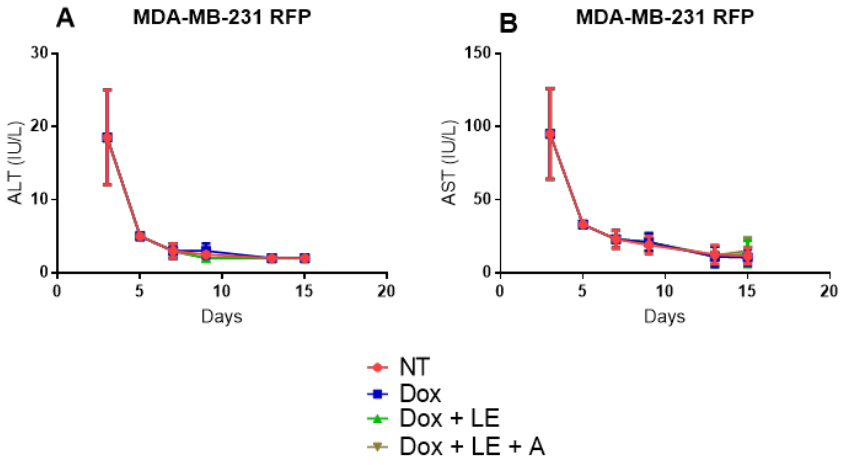


Figure 56. Atorvastatin does not affect hepatocyte health in the MPS

Supernatant from MPS wells containing hepatocyte and non-parenchymal cells cultured with MDA-MB-231 RFP were tested for leakage of (A) ALT and (B) AST using assays performed in clinical chemistry laboratories at the University of Pittsburgh Medical Center (UPMC). The initially high markers of hepatocyte damage reflect the isolation protocols. NT = no treatment, Dox = 1µM doxorubicin for days 7-10, LE = 1µg/mL LPS + 20ng/mL EGF for days 13-15, and A = 5µM atorvastatin for days 11-15. Error bars represent the standard error of the mean (n=2).

To better characterize the primary tumor and metastases in the spleen-to-liver mouse model of spontaneous breast cancer metastasis, E-cadherin immunohistochemistry was performed. Similar to *in vitro*, the primary tumors were marked by a lack of E-cadherin expression (Figure 57A,B). While small micrometastases exhibited membrane E-cadherin expression, larger metastases (>100μm) were largely E-cadherin negative (Figure 57C,D), having undergone a second EMT[748,965].

To assess for tumor cell death, TUNEL staining was performed. Primary splenic tumors exhibited minimal TUNEL staining (Figure 58A,B) that was unaffected by 10mg/kg atorvastatin treatment (Figure 58C). Similarly, liver metastases stained negative for TUNEL (Figure 58D,E) that was unaffected by 10mg/kg atorvastatin treatment (Figure 58F). These data suggest that cytotoxic effects of atorvastatin were minimal in this mouse model for breast cancer metastasis.

8.5 CONCLUSIONS

This study adds to the previous study by examining the growth suppressive effects of statins in models of metastatic breast cancer of increasing complexity. In breast cancer 2D co-culture with primary human hepatocytes, the lipophilic atorvastatin remains more effective than the hydrophilic rosuvastatin (Figure 47). Similarly, in this co-culture system, the synergy of inhibiting the PI3K-Akt pathway was preserved (Figure 48). In a 3D MPS model of breast cancer metastases in the liver, atorvastatin could significantly suppress LPS/EGF stimulated outgrowth of breast cancer cells (Figure 49). Finally, in two independent models for spontaneous breast cancer metastasis to the liver and lung, respectively, atorvastatin was able to suppress proliferation of metastatic but not primary tumor cells (Figures 50-54).

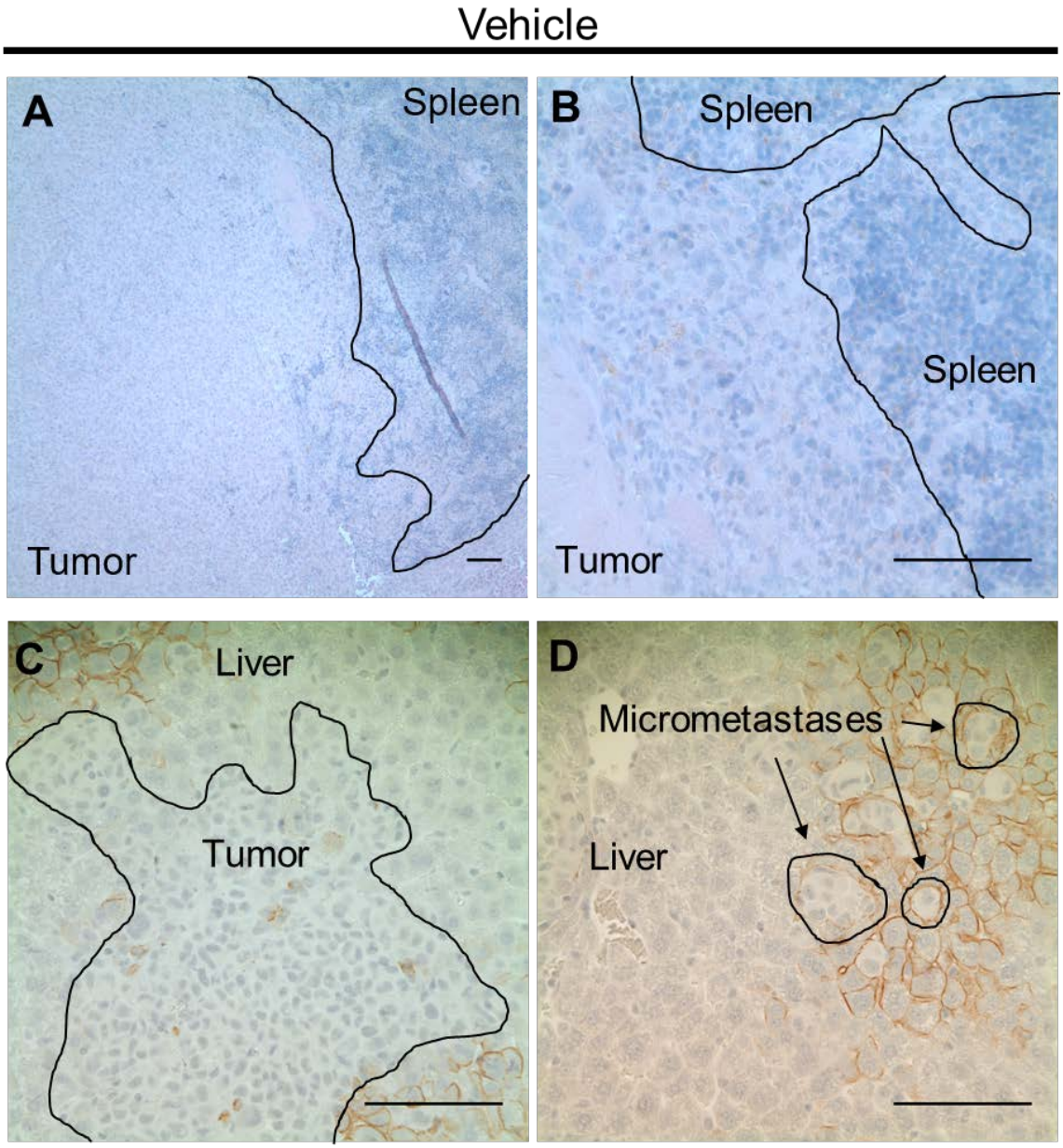


Figure 57. MDA-MB-231 RFP cells re-express E-cadherin in small liver micrometastases

MDA-MB-231 RFP cells in the intrasplenic inoculation model of breast cancer metastasis to liver (Figure 50, Figure 51) were assessed for E-cadherin expression in (A,B) the splenic primary tumor and (C) large or (D) small liver metastases. Scale bar = 100µm

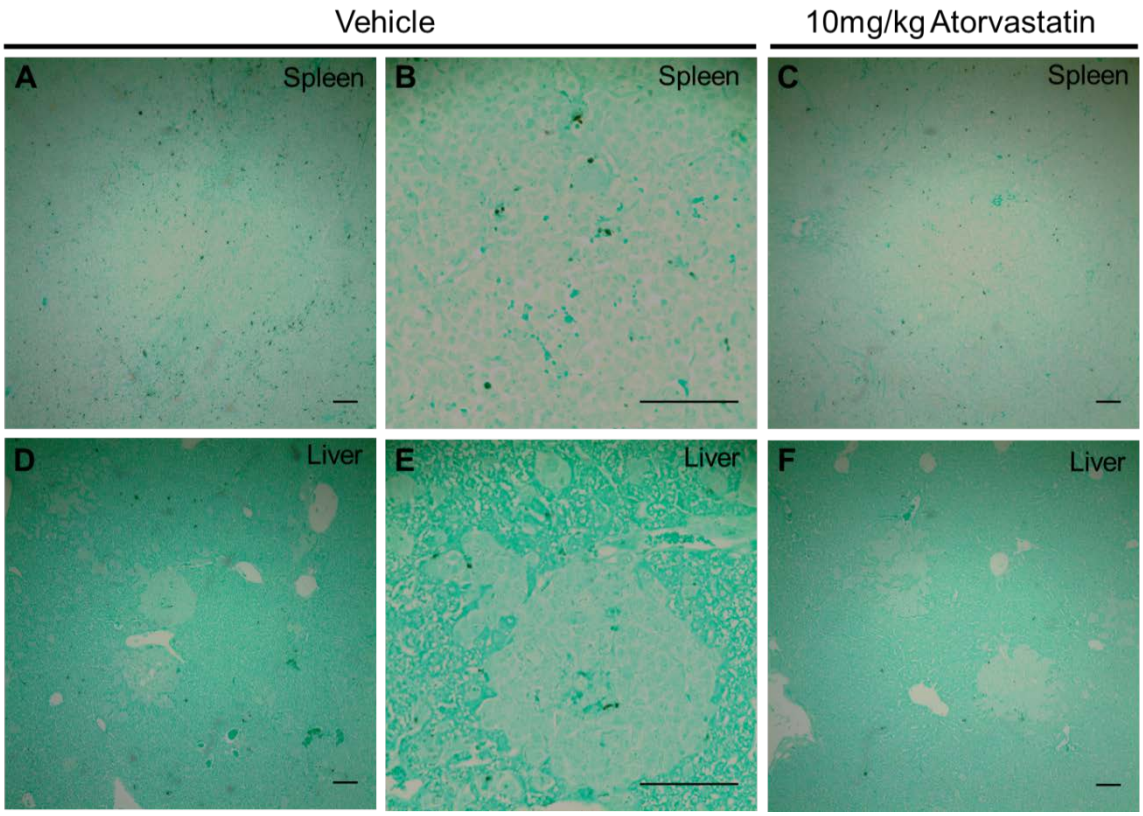


Figure 58. Atorvastatin is not cytotoxic to MDA-MB-231 primary tumor or metastatic cells

MDA-MB-231 RFP cells in the intrasplenic inoculation model of breast cancer metastasis to liver (Figure 50, Figure 51) were assessed for cell death by TUNEL staining. Cell death was assessed for (A,B,D,E) vehicle and (C,F) 10mg/kg atorvastatin treatment in (A-C) the splenic primary tumor and (D-F) liver metastases. Scale bar = 100µm

The *in vivo* data (Figures 50-54) are the most significant contribution of this study to the overall hypothesis of this thesis. First, the doses of atorvastatin used in both mouse models are similar to those used for moderate-intensity lipid lowering therapy in clinical patients[607]. Accounting for the difference in volume of distribution, the 2mg/kg and 10mg/kg doses in mice are approximately equivalent to 20mg and 80mg doses in human patients[966]. Thus, the doses of atorvastatin that suppress metastatic proliferation are similar to those already employed clinically with little systemic toxicity. Second, we found a divergent effect of atorvastatin on breast cancer

cells. While the primary tumor cells were unaffected by statin treatment, the metastatic cells were suppressed. This was found in our two mouse models and in our MPS model of breast cancer metastasis to the liver (Figures 49-54). These results are similar to other reports that statins can delay the formation of metastases in mice[743,967]. Breast cancer cells are known to re-express E-cadherin and enter a period of quiescence at the metastatic site[45]. Since atorvastatin was able to suppress stimulated outgrowth of dormant MDA-MB-231 cells (Figure 49), this suggests that atorvastatin can suppress emergence of dormant tumor cells.

The divergent effects of atorvastatin on the primary and metastatic tumor cells is supported by the clinical data suggesting a mortality but not incidence benefit of statins in breast cancer[531,532]. Other studies have reported statin suppression of primary tumor growth and induction of apoptosis in mouse models[537,968]. However, previous studies have not compared proliferation at the primary and metastatic sites and used doses higher than those used for cholesterol-lowering. Our data suggest atorvastatin can preferentially suppress metastatic breast cancer outgrowth and as such, may be useful as a long term adjuvant for preventing emergence of dormant breast cancer micrometastases that eventually progress to clinically evident disease. Since the concentrations needed to achieve these anti-metastasis effects are currently seen with clinical use in cardiovascular disease, targeted clinical trials of adjuvant statin therapy in breast cancer patients at risk for metastatic recurrence may improve mortality without causing significant toxicity. Moreover, atorvastatin efficacy should be examined in metastasis models of other cancer types, such as metastatic prostate cancer.

9.0 CLINICAL IMPLICATIONS

There are several critical insights to draw from these research findings. First, statin drugs have differential effects on tumor cells depending on their potency and lipophilicity. The fact that the lipophilic atorvastatin and simvastatin are more potent at suppressing cancer cell growth is critical to investigating statins as anti-cancer agents. The results of the present work suggest that women with a history of breast cancer who are currently taking a hydrophilic statin for cardiovascular indications should be switched to a lipophilic statin. Approximately 20% of patients taking statins receive a hydrophilic statin, which suggests there is a significant portion of the female breast cancer population that would benefit from this therapeutic switch. Additionally, all future clinical trials that investigate statins in cancer therapy should only be conducted using lipophilic statins, as the present work has proven that hydrophilic statins are ineffective for cancer treatment.

Second, statins have different effects on primary and metastatic tumor cells, because they suppress proliferation of metastases while having no effect on growth of the primary tumor. The inefficacy of statins against primary tumor cells suggests they are not useful as neoadjuvant therapy. In contrast, these data motivate statin use as an adjuvant therapy to prevent tumor recurrence. Indeed, the largest retrospective cohort studies suggest statins benefit tumor recurrence and mortality[531,664]. Most importantly, atorvastatin was shown to block stimulated outgrowth of dormant tumor cells, which suggests statins can directly block tumor recurrence in the context of the liver microenvironment.

Third, transcriptomic analysis as used in the present work can reveal candidate genes that are involved with susceptibility to statin-mediated growth suppression. Moreover, drugs expected to synergize with statins can be predicted using these advanced computational models. While both

these candidate biomarkers and synergistic drugs need experimental validation, the present work lays a foundation for discovery and validation of factors that can be expected to potentiate statin efficacy in human cancer patients or identify the patients who are most likely to respond to statin therapy.

Finally, this work reveals important signaling networks that contribute to statin sensitivity. In the breast cancer cell lines tested, a reduction in Ras signaling through PI3K-Akt was found to be responsible for statin-mediated growth suppression. Since PI3K and Akt inhibitors have shown significant clinical toxicity, combination therapy with statins may allow for a lower dose of these drugs to be used to achieve similar efficacy with a reduction in toxicity.

10.0 FUTURE DIRECTIONS

Further investigation of statins as anti-tumor agents will encompass both basic research and clinical research studies. Promising avenues of research indicated by the results of the present work are described below.

10.1 BASIC RESEARCH STUDIES

The present work has discovered mechanistic and pharmacologic insight into the use of statins as anti-tumor agents, but additional questions remain regarding their use in breast cancer. First, the efficacy of statins against breast cancer cells with different molecular subtypes is not yet well understood. The breast cancer cell lines used for the majority of this work were either hormone receptor positive (MCF-7) or triple-negative (MDA-MB-231). While MDA-MB-231 cells were found to be more sensitive to statin therapy, it is not known whether this holds true for all triple-negative breast cancer cell lines. The susceptibility of many breast cancer cell lines – hormone receptor positive, HER2 positive, and triple-negative – should be investigated in order to determine whether molecular subtype plays a role in statin sensitivity. Moreover, patient-derived cells should be tested to validate the cell line data.

Second, the difference in efficacy of statins against primary and metastatic tumor cells should be further investigated. It is not yet known whether human metastasis, which often occurs over much longer time scales, will be reflected by the results of the mouse models described herein. The evolution of tumor cells between the primary and metastatic tumor may explain the selectivity

of statins for the latter. In order to better investigate the differential potency of statins against primary and metastatic tumor cells, paired biopsy samples from clinical patients should be used in the *in vitro*, *ex vivo*, and *in vivo* models described in this thesis. This may reveal the relative importance of intrinsic differences in statin susceptibility of the primary and metastatic cells and the differences in the primary and metastatic microenvironments. Moreover, there may be a difference in statin susceptibility with the site of metastasis. Indeed, the mouse models of spontaneous breast cancer metastasis used in this work demonstrated a more potent effect of statins against metastatic cells that migrated to liver as compared to lung. Understanding the site dependence of statin anti-tumor efficacy is critical in determining which patients may benefit the most from their use.

Third, it is critical to understand how statins interact with currently used breast cancer therapies, as statin use may either enhance or diminish their efficacy. For example, since statins work by inducing growth arrest and decreasing proliferation, the efficacy of chemotherapeutic agents may be compromised since they are only effective against cycling cells. If the findings from the mouse models hold true for human disease, patients should stop taking their statin during chemotherapy cycles. In contrast, statins have been shown to induce oxidative stress in cancer cells, which may compromise the health of tumor cells and make them more susceptible to cytotoxic drugs. If these findings hold true in humans, patients who don't normally take statins should supplement their chemotherapy regimen with a lipophilic statin. Finally, since most statins are metabolized by CYP450 isozymes, most commonly CYP3A4 and CYP2C9, they may interfere with the metabolism of chemotherapeutic drugs and possibly induce additional adverse events. Examining statins in combination with other chemotherapeutic drugs in *ex vivo* liver culture

systems and *in vivo* will suggest which drug combinations may result in higher clinical efficacy and toxicity.

Finally, there is a need for a continued systematic search for existing drugs and novel compounds that can potentiate the growth inhibitory effect of lipophilic statins. The sophisticated tools available for this discovery process included unbiased screening, bioinformatics, network biology, and hypothesis-driven approaches. Chapter 7 of this dissertation has already demonstrated how powerful and successful these computational approaches can be. After identifying candidate compounds, these drugs should be tested in combination with statins in experimental systems (such as the *ex vivo* and *in vivo* models discussed in this dissertation) to determine the efficacy and/or limitations of these approaches. These studies will pave the way for clinical translation of promising combination therapies to reduce the morbidity and mortality of breast cancer.

10.2 CLINICAL RESEARCH STUDIES

Since statins have been shown to be effective primarily against metastatic tumor cells, prospective clinical trials should investigate their use as adjuvant therapy in breast cancer. Since the studies that demonstrate a cancer mortality benefit with statin use are quite large, prospective clinical trials would need to be conducted in a targeted patient population. For example, investigating adjuvant statin therapy in triple-negative or HER2-positive patients would be more feasible than a trial of hormone receptor positive patients since the former two groups typically relapse earlier. Moreover, patients with nodal metastases or locally invasive primary tumors would be prime candidates for these trials, as they are at the highest risk for metastatic relapse. Patients would be randomized to a lipophilic statin (likely atorvastatin) or placebo and followed longitudinally. These trials would

contain defined interim analysis periods to allow patients to crossover from placebo to statin should the statin recurrence or mortality benefits prove significant.

10.3 CONCLUSIONS

Future basic research studies and clinical trials that build upon the results of the present work have the potential to establish statins as effective and safe agents to delay breast cancer recurrence and mortality. In the short term, studies should address whether current statin users for cardiovascular indications with a history of breast cancer should be uniformly prescribed lipophilic statins. In parallel, basic studies should determine how statins interact with the efficacy and toxicity of clinically used breast cancer treatments. In the long term, clinical trials should address whether statins play a role in adjuvant management of breast cancer in women who do not otherwise require a statin. It is hoped that accomplishing both of these short and long term goals will improve the quality of life and survival rates of women with breast cancer.

APPENDIX

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- GPA: 3.975 (on a 4.0 scale)
- *Thesis Title:* Breast cancer metastatic dormancy and emergence, a role for adjuvant statin therapy; *Advisor:* Dr. Alan Wells MD DMSc
- *Oral Thesis Examination:* Pass with Honors, 5/22/18

MD, Expected Graduation: May, 2020

- USMLE Step 1: 254

Massachusetts Institute of Technology, Boston, MA (2009 – 2013)

BS, Biological Engineering: May, 2013

- GPA: 5.0 (on a 5.0 scale)

Professional Society Membership, Leadership, and Committee Involvement

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University of Pittsburgh MSTP Screening Committee: 2017 – Present
Social Co-Chair for the MIT-Pittsburgh Alumni Club: 2016 - Present
Cellular and Molecular Pathology Representative: 2016 – 2017
University of Pittsburgh MSTP Interviewing Committee: 2015 – 2017
PalPittations, Medical School A Cappella Group, Music Director: 2014 – 2015
American Medical Association, Medical Student Member: 2013 – 2017
University of Pittsburgh MSTP Second Look Committee: 2013 – 2014

Honors and Professional Awards

University of Pittsburgh Department of Pathology Retreat, 2nd place poster: May 23rd, 2018
Cellular and Molecular Pathology PhD Thesis Defense, Pass with Honors: May 22nd, 2018
VA Research Week Poster Award, First Place Winner: May 14th, 2018
McGowan Institute of Regenerative Medicine Annual Retreat, 2nd place poster: March 6th, 2018

Individual Pre-doctoral National Research Service Award: February 23rd, 2018
Biomedical Graduate Students Association Symposium, Best Poster Award: October 11th, 2017
McGowan Institute of Regenerative Medicine Annual Retreat, 3rd place poster: March 6th, 2017
University of Pittsburgh Department of Pathology Retreat, 3rd place poster: May 25th, 2016
Autopsy Discovery Program, 3rd place poster: March 3rd, 2015
Phi Beta Kappa, honors society: June 6th, 2013
Outstanding Contribution to the MIT Biological Engineering Community: June 5th, 2013

Funding Awards

National Cancer Institute Pre-doctoral Fellowship, F30 Grant: September 2016 – Present
University of Pittsburgh CATER Training Program, T32 Grant: September 2015 – August 2016
University of Pittsburgh Medical Scientist Training Program, T32 Grant: June 2013 – May 2015

Research and Laboratory Experiences

Graduate Student Researcher, *University of Pittsburgh*, summer 2014 and 2015 – Present
Investigating the application of statin drugs as adjuvants for the treatment of metastatic breast cancer. Advisor: Alan Wells MD, DMSc

Medical Student Researcher, *University of Pittsburgh*, 2016 – Present
Investigating primary ankle arthrodesis versus primary ORIF in AO/OTA type C3 pilon fractures. **Advisor:** Gary Gruen MD

Medical Student Researcher, *University of Pittsburgh*, February – March 2015
Pheochromocytoma: a catecholamine-secreting neuroendocrine tumor of the adrenal medulla. **Advisor:** Jeffery Nine MD

Graduate Student Researcher, *University of Pittsburgh*, summer 2013
Investigating the role of CD47 in promoting hepatocyte survival. **Advisor:** Steven Badylak DVM MD PhD

Undergraduate Student Researcher, *Massachusetts Institute of Technology*, 2011 – 2012
Multilayer assembly of mucin – lectin biofilms for drug delivery. **Advisor:** Katharina Ribbeck PhD

Undergraduate Student Researcher, *Massachusetts Institute of Technology*, summer 2010
Assembly and characterization of a Thorlabs optical trap kit. **Advisor:** Steven Wasserman PhD

Undergraduate Student Researcher, *Massachusetts Institute of Technology*, summer 2009
Modular design of a multi-sensor housing cube for experimental physics applications. **Advisor:** Ian Hunter PhD

Summer Research Instructor, *Massachusetts Institute of Technology*, summers 2008 – 2011
Measuring biological forces through use of an optical trap. **Advisor:** Steven Wasserman PhD

Peer-Reviewed Publications

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1. **Beckwitt CH**, Shiraha K, and Wells A. Lipophilic statins limit cancer cell growth and survival, via involvement of Akt signaling. PLoS One, 2018, May 15; 13(5): e0197422
2. Ishikawa T, Hosaka YZ, **Beckwitt CH**, Wells A, Oltvai ZN, and Warita K. Attenuation of HMG-CoA reductase expression potentiates the cancer cell growth-inhibitory effect of statins. *Oncotarget, accepted 5/1/18*

3. **Beckwitt CH**, Monaco SJ, Gruen GS. Primary arthrodesis of the tibiotalar joint achieves comparable outcomes to ORIF in severely comminuted high-energy pilon fractures: a retrospective cohort study. *Foot and Ankle Orthopedics, accepted 4/14/18*
4. **Beckwitt CH**, Clark A, Wheeler S, Stolz DB, Taylor Lans, Griffith L, Wells A. Liver "Organ on a Chip". Exp Cell Res, 2018 Feb 1; 363(1): 15-25
5. Raghu V, **Beckwitt CH**, Warita K, Wells A, Benos PV, and Oltvai ZN. Biomarker identification for statin sensitivity of cancer cell lines and tumors. Biochem Biophys Res Commun, 2018 Jan 1; 495(1): 659-665
6. Dioufa N, Clark AM, Ma B, **Beckwitt CH**, and Wells A. Bi-directional exosome-driven intercommunication between the hepatic niche and cancer cells. Molecular Cancer, 2017 Nov 14, 16:172
7. Warita K, Warita T, **Beckwitt CH**, Schurdak ME, Vazquez A, Wells A, Oltvai ZN. Statin-induced mevalonate pathway inhibition attenuates the growth of mesenchymal-like cancer cells that lack functional E-cadherin mediated cell cohesion. Sci Rep, 2014 Dec 23; 4: 7593
8. Crouzier T, **Beckwitt CH**, Ribbeck K. Mucin multilayers assembled through sugar-lectin interactions. Biomacromolecules. 2012 Oct 8;13(10): 3401-8.

Publications in Review

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1. **Beckwitt CH**, Brufsky AM, Oltvai ZN, and Wells A. Statin drugs to reduce breast cancer recurrence and mortality. *In review at Breast Cancer Research, submitted 5/24/18*
2. **Beckwitt CH**, Clark AM, Ma B, Whaley D, Oltvai ZN, and Wells A. Statins attenuate outgrowth of breast cancer metastases. *In review at British Journal of Cancer, submitted 5/9/18*

National Poster Presentations

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1. Adjuvant Statin Therapy Efficacy is dictated by Tumor Dormancy and Statin Lipophilicity in ex vivo and in vivo Models of Metastatic Breast Cancer. **Beckwitt CH**, Warita K, Clark AM, Oltvai ZN, and Wells A. December 2017: San Antonio Breast Cancer Symposium, San Antonio, Texas
2. Adjuvant Statin Therapy Efficacy is dictated by Tumor Dormancy and Statin Lipophilicity in in vitro and ex vivo Models of Metastatic Breast Cancer. **Beckwitt CH**, Warita K, Clark AM, Oltvai ZN, and Wells A. April 2017: Experimental Biology 2017 Conference, Chicago, IL
3. A MPS that models dormant-emergent metastatic breast cancer progression. Clark AM, Wheeler SE, Young CL, **Beckwitt CH**, Ma B, Bradshaw AM, Wang A, Edington C, Shepard J, Chen K, Geishecker E, Stockdale L, Zhao W, Trumper D, Stolz DB, Venkataramanan R, Griffith LG, Wells A. September 2016: Microphysiological Systems Review Meeting, DARPA, Arlington, VA
4. Breast Cancer Dormancy, Re-emergance, and Treatment. Wheeler SE, Clark AM, Young CL, Pillai VC, **Beckwitt CH**, Stolz DB, Lauffenburger DA, Venkataramanan R, Griffith LG, Wells A. December 2015: San Antonio Breast Cancer Symposium, San Antonio, Texas

Local Poster Presentations

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1. Atorvastatin inhibits outgrowth of breast cancer metastases. **Beckwitt CH**, Warita K, Clark AM, Oltvai ZN, and Wells A. May 2018: VA Research Week, VA Pittsburgh Healthcare System, Pittsburgh, PA (**1st Place**); May 2018: CMP Program Retreat, University of Pittsburgh, Pittsburgh, PA, (**2nd Place**)
2. Adjuvant Statin Therapy Efficacy is dictated by Tumor Dormancy and Statin Lipophilicity in ex vivo and in vivo Models of Metastatic Breast Cancer. **Beckwitt CH**, Warita K, Clark AM, Oltvai ZN, and Wells A. February 2018: IGBP orientation, University of Pittsburgh, Pittsburgh, PA; March 2018: McGowan Annual Retreat, Pittsburgh, PA (**2nd Place**)
3. Adjuvant Statin Therapy Efficacy is dictated by Tumor Dormancy and Statin Lipophilicity in in vitro and ex vivo Models of Metastatic Breast Cancer. **Beckwitt CH**, Warita K, Clark AM, Oltvai ZN, and Wells A. June 2017: UPCI Annual Retreat, Pittsburgh, PA; August 2017: IGBP orientation, University of Pittsburgh, Pittsburgh, PA; October 2017: BGSA Symposium, University of Pittsburgh, Pittsburgh, PA (**Best Poster Award**, 4 out of 60 total)
4. Statins suppress breast and other cancer cell growth in the context of the metastatic microenvironment. **Beckwitt CH**, Warita K, Clark AM, Hosaka YZ, Oltvai ZN, and Wells A. October 2016: BGSA Symposium, University of Pittsburgh, Pittsburgh, PA; March 2017: McGowan Annual Retreat, Nemacolin, PA, (**3rd Place**)
5. Statin inhibition of cancer cell growth tracks with lack of functional E-cadherin mediated cell cohesion and is dependent on statin pharmacologic properties. **Beckwitt CH***, Warita K*, Warita T, Schurdak ME, Clark A, Wheeler S, Vazquez A, Wells A, Oltvai ZN. May 2016: CMP Program Retreat, University of Pittsburgh, Pittsburgh, PA, (3rd Place)
6. Pheochromocytoma: A Catecholamine-secreting Neuroendocrine Tumor of the Adrenal Medulla. **Beckwitt CH**, Trejo Bittar HE, Nine J. March 2015: Autopsy Discovery Program Poster Session, University of Pittsburgh, Pittsburgh, PA, (**3rd place**)
7. Statin inhibition of cancer cell growth tracks with mesenchymal phenotype and lack of functional E-cadherin mediated cell cohesion. **Beckwitt CH***, Warita K*, Warita T, Schurdak ME, Clark A, Wheeler S, Vazquez A, Wells A, Oltvai ZN. August 2014: Pitt MSTP Program Retreat, University of Pittsburgh, Pittsburgh, PA
8. Mucin multilayers assembled through sugar-lectin interactions. **Beckwitt CH**, Crouzier T, Ribbeck K. March, 2013: MIT Biological Engineering Graduate School poster session, MIT, Cambridge, MA

Local Oral Presentations

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1. Atorvastatin inhibits outgrowth of breast cancer metastases. CMP Retreat, May 23, 2018
2. Breast cancer metastatic dormancy and emergence, a role for adjuvant statin therapy. CATER Training Program Lunch Seminar, March 23, 2018
3. Statins Suppress Growth of Metastatic Breast Cancer in the Context of the Liver Microenvironment. Pitt MSTP retreat, August 25, 2017
4. Statin Mediated Suppression of Breast Cancer Growth. Department of Pathology Lunch Seminar, Dec 7, 2016
5. Statin-Mediated Suppression of Tumor Cell Growth. CATER Training Program Lunch Seminar, October 14, 2016
6. Statin-Mediated Suppression of Cancer Cell Growth. Pitt MSTP retreat, August 19, 2016.

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1. Beckwitt CH, Clark AM, Wheeler S, et al. Liver “organ on a chip”. *Exp Cell Res*. 2018;363(1):15-25. doi:10.1016/j.yexcr.2017.12.023.

2. Warita K, Warita T, Beckwitt CH, et al. Statin-induced mevalonate pathway inhibition attenuates the growth of mesenchymal-like cancer cells that lack functional E-cadherin mediated cell cohesion. *Sci Rep*. 2014;4:7593. doi:10.1038/srep07593.

3. Raghu VK, Beckwitt CH, Warita K, Wells A, Benos P V, Oltvai ZN. Biomarker identification for statin sensitivity of cancer cell lines. *Biochem Biophys Res Commun*. 2018;495(1):659-665. doi:10.1016/j.bbrc.2017.11.065.

4. Beckwitt CH, Shiraha K, Wells A. Lipophilic statins limit cancer cell growth and survival, via involvement of Akt signaling. *PLoS One*. 2018;13(5):e0197422. doi:10.1371/journal.pone.0197422.

5. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. *CA Cancer J Clin*. 2018;68(1):7-30. doi:10.3322/caac.21442.

6. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2017. *CA Cancer J Clin*. 2017;67(1):7-30. doi:10.3322/caac.21387.

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